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The Combination between Methæmoglobin and Peroxides: Hydrogen Peroxide and Ethyl Hydroperoxide

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INTRODUCTION

It was shown by Kobert in 1900 that when hydrogen peroxide is added to a solution of acid methæmoglobin, the solution turns from brown to red and the characteristic absorption spectrum of methæmoglobin is replaced by three diffuse bands at 600–584 mμ, 558–545 mμ, 513–500 mμ.* This solution, on standing, and especially on warming, turns brown again and its absorption spectrum rapidly reverts to that of the ordinary acid methæmoglobin. Kobert concluded that the red solution thus obtained is a loose combination between methæmoglobin and H_2O_2 which he called " Wasserstoffsuperoxydmethemoglobin."

Kobert's observation was confirmed only in 1924 by Haurowitz, who noticed that acid methæmoglobin on addition of H_2O_2 changes its colour and its absorption spectrum. He did not, however, ascribe this change to the formation of H_2O_2-methæmoglobin but to the transformation of acid methæmoglobin into alkaline methæmoglobin (p. 25). In a later paper in 1931 Haurowitz repeated his observations, using this time a recrystallized preparation of methæmoglobin, and found that: " the two banded absorption spectrum of this solution remained unchanged on the addition of potassium ferricyanide or of sodium cyanide; also a great excess of $Na_2S_2O_4$ did not bring about the appearance of the absorption bands of hæmoglobin, while methæmoglobin and its derivatives when treated in this way are easily transformed into hæmoglobin. The spectroscopic appearance of the preparation resembles also that of NO-hæmoglobin." According to this statement the methæmoglobin treated with H_2O_2 does not seem to form a H_2O_2-methæmoglobin as was postulated by Kobert.

* The third band, as will be shown, belongs to the free unbound acid methæmoglobin.

The object of this investigation is to show (1) that, contrary to the views expressed by Haurowitz, methæmoglobin combines with H_2O_2 in the same way as it does with KCN, H_2S, KF, and NaN_3, and that the formation of the compound can be followed quantitatively; and (2) that methæmoglobin also forms a similar compound with an organic peroxide, namely, ethyl hydroperoxide (C_2H_5OOH).

PREPARATION OF METHÆMOGLOBIN

Methæmoglobin solution prepared from washed and laked blood corpuscles, being very rich in catalase, cannot be used directly in this investigation. Hydrogen peroxide added to such a solution is rapidly decomposed, filling the solution with bubbles of oxygen. Methæmoglobin solution with practically no catalase, or a very reduced catalase concentration, suitable for this investigation was prepared in the following way.

Fresh horse blood is defibrinated, the red blood corpuscles are separated from serum and washed three times with 0·9% NaCl. 300 cc of red blood corpuscles thus obtained are cooled in the ice chest and laked with 90 cc of cold distilled water and 90 cc of cold ether previously washed with N . NaOH. The mixture is shaken for 5 minutes and centrifuged for 5–10 minutes. The centrifuge tube shows two distinct layers, a thick and more or less solid layer of stroma on the top and a clear, strong solution of hæmoglobin underneath. The centrifuge tube is slightly tilted, causing the solid cake to float to one side, and the clear hæmoglobin solution is sucked off. The solution is rapidly filtered, cooled, and mixed with ice-cold alcohol which is added very slowly until its total concentration reaches about 20%. The mixture is put into the ice chest where, after 2 or 3 hours, the crystallization of hæmoglobin begins and is completed when left overnight. The crystals are centrifuged off in cooled tubes, washed with ice-cold 20% alcohol and then in ice-cold distilled water. During the last manipulation some of the hæmoglobin is redissolved. The crystals are collected, mixed with an equal volume of distilled water and warmed to 39° C. On addition of 10 cc of N/10 NaOH per 100 cc of such suspension, which brings the mixture to p_H 7·63, the crystals rapidly dissolve. The solution is neutralized with N/10 HCl to p_H 6·9–7·1, centrifuged to remove a slight precipitate and left overnight in the ice chest where it crystallizes again to a solid mass. It is again redissolved and recrystallized three or four times. Finally the solution is dialysed against distilled water and its concentration is estimated by the usual methods. In this way various solutions containing up to 18% of hæmoglobin but very poor in catalase are easily obtained.

To prepare methæmoglobin a strong solution of hæmoglobin thus obtained is treated with the minimum amount of potassium ferricyanide

until it is completely converted into acid methæmoglobin. It is then electrodialysed for 4–5 hours against running tap water, filtered and dialysed overnight against distilled water.

The concentration of this methæmoglobin solution was determined spectroscopically by comparing it with methæmoglobin freshly prepared from a hæmoglobin of known concentration. The values obtained in this way agreed well with those obtained by the determination of the protein content of the preparation.*

COMBINATION BETWEEN METHÆMOGLOBIN AND H_2O_2

Evidence of the Formation of H_2O_2-*methæmoglobin*—Pure methæmoglobin solution prepared as described above and acidified with KH_2PO_4 to p_H 5·8–6·5 is of distinctly brown colour and shows a typical absorption spectrum of acid methæmoglobin. On addition of a little hydrogen peroxide it turns red and the absorption spectrum of acid methæmoglobin is replaced by a spectrum composed of two diffuse bands. The first absorption band, which lies at about 589 mμ, is unsymmetrical, being darker in the region nearer the red end of the spectrum. It has a slight indication of being double, *i.e.*, having two maxima. The second band is more symmetrical and stronger than the first band and has its centre at about 545 mμ. The absorption spectrum of this compound shows only these two bands; the third band described by Kobert (at 513–500 mμ) does not belong to this compound but to the free acid methæmoglobin which has its strongest absorption band in this position. This band is visible only in the presence of some free, uncombined methæmoglobin or when the completely formed compound is partly decomposed liberating a small amount of acid methæmoglobin.

That the two-banded spectrum is not due to the transformation of acid into alkaline methæmoglobin, as was at first suggested by Haurowitz, is obvious from the fact that the solution remains distinctly acid with p_H varying from 5·8 to 6·5. At this p_H methæmoglobin solution exists only in its acid form. Moreover, the absorption spectrum of this compound resembles only superficially that of an alkaline methæmoglobin. It can also be demonstrated that the formation of the red compound is due to the combination of H_2O_2 with methæmoglobin (ferric compound) and not to its combination with hæmoglobin (ferrous compound) as was more recently suggested by Haurowitz. In fact, while the addition of H_2O_2 rapidly changes the colour and absorption spectrum of methæmoglobin in the presence of some catalase, it has no effect under the same conditions

* Refractometric determinations of protein content were kindly carried out by Mr. G. S. Adair.

on reduced hæmoglobin, oxyhæmoglobin, or CO-hæmoglobin. In the absence of catalase, H_2O_2 transforms the ferrous into ferric compound and then combines with the latter. Moreover, while the red compound. is not affected by potassium ferricyanide, it is rapidly transformed into reduced hæmoglobin by the addition of $Na_2S_2O_4$.

The H_2O_2-methæmoglobin compound is not stable and even at room temperature it gradually undergoes decomposition, liberating acid methæmoglobin (see p. 10).

Quantitative Spectroscopic Study of the H_2O_2-methæmoglobin Formation—In order to determine the minimum amount of H_2O_2 which is required to convert acid methæmoglobin into H_2O_2-methæmoglobin we have adopted the same method which we have previously used in our investigation on H_2S-methæmoglobin (Keilin, 1933, *a*, *b*). For this purpose acid methæmoglobin and H_2O_2 are mixed in various concentrations in reaction vessels and the relative concentrations of free acid methæmoglobin and H_2O_2-methæmoglobin formed are determined by comparing the absorption spectrum of the mixtures with that given by the two substances, acid methæmoglobin and H_2O_2-methæmoglobin in equal concentrations but kept separately in the two compartments of a double wedge trough. The examination is carried out by means of a Zeiss' spectroscope ocular attached to the microscope. For the reaction special vessels of Zeiss' comparative spectroscope provided with movable plungers protected with paraffin are placed on the microscope stage. The double wedge trough 150 mm long and 23 mm wide is placed in front of the aperture of the comparison prism on a brass platform attached to the microscope tube. The position of the trough is read off on a scale fixed to the platform. The light during observation must be adjusted, as was shown previously, in such a way that in only one position of the trough as it moves along the scale the two absorption spectra match each other over the whole length of the visible spectrum. From the reading of this position of the trough, the relative concentrations of both compounds in the reaction vessels are easily calculated.

A typical experiment is carried out as follows: both compartments of the trough receive acid methæmoglobin $1 \cdot 3 \times 10^{-4}$ (gm atom of Fe per l.), and one of them receives in addition $0 \cdot 1$ cc of $0 \cdot 143$ M H_2O_2, which transforms it completely into H_2O_2-methæmoglobin. The solutions for the reaction vessels are prepared in six tubes, each receiving 3 cc of acid methæmoglobin 6×10^{-4} (gm atom Fe per l.) together with gradually increasing amounts ($0 \cdot 1$ to $0 \cdot 6$ cc) of 6×10^{-3} M H_2O_2, the contents of the tubes being made up to $3 \cdot 6$ cc with water.

In these experiments H_2O_2 is added just before the observation, and the examination has to be carried out very rapidly, because the compound being unstable undergoes decomposition, liberating acid methæmoglobin. The final concentrations of the solutions and the results of these experiments are shown in Table I and fig. 1.

These experiments clearly show (fig. 1) that the minimum amount of H_2O_2 required to transform acid methæmoglobin into H_2O_2-methæmoglobin correspond to 1 molecule of H_2O_2 per iron atom of methæmoglobin.

In spite of the experimental difficulties due (1) to the presence of traces of catalase, and (2) to the instability of H_2O_2-methæmoglobin compound, the experimental curves, I and II, fig. 1, are found to be surprisingly near the theoretical curve for the formation of a compound having one molecule of H_2O_2 per atom of Fe of methæmoglobin.

Manometric Study of the Formation of H_2O_2-*methæmoglobin*—The formation of H_2O_2-methæmoglobin can also be demonstrated manometrically. The principle of this method consists in mixing in a flask on one side of a Barcroft differential manometer a definite amount of H_2O_2 with catalase preparation in presence of either ordinary buffer solution or a strong solution of methæmoglobin, CO-hæmoglobin, or oxyhæmoglobin. A strong preparation of catalase in water or buffer solution when mixed in a Barcroft flask with H_2O_2 rapidly decomposes it, liberating almost an equivalent amount of oxygen, which can be estimated from the reading of the manometer. Approximately the same amount of oxygen is liberated from H_2O_2 by catalase in presence of the solution of CO-hæmoglobin saturated with CO. On the contrary, in the presence of methæmoglobin the amount of which in terms of Fe is equal to that of H_2O_2 (Fe/$H_2O_2 = 1$), very little oxygen is liberated because in this case most of the H_2O_2 added combines with methæmoglobin which protects it from decomposition by catalase. In the presence of oxyhæmoglobin the reaction is more complicated especially when the concentration of catalase is sufficiently low. In this case one fraction of the H_2O_2 is decomposed by catalase, liberating the corresponding amount of oxygen; the other fraction which is used in the oxidation of the divalent iron of oxyhæmoglobin to the trivalent iron of methæmoglobin does not yield oxygen directly, but liberates the oxygen from oxyhæmoglobin, and finally a small fraction of the H_2O_2 is united with methæmoglobin and is therefore protected from decomposition by catalase,* fig. 3.

* The oxidation of hæmoglobin to methæmoglobin by H_2O_2 will be discussed later.
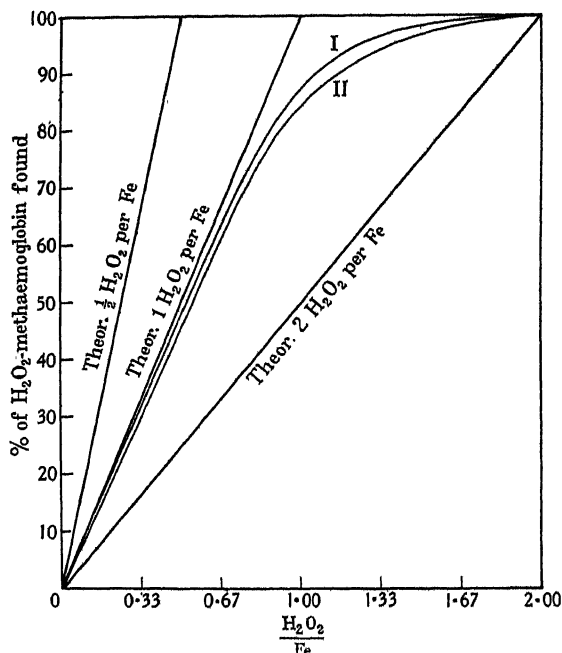
FIG. 1—Formation of H_2O_2-methæmoglobin showing that the two experimental curves (I and II) lie very near the theoretical curve of one molecule of H_2O_2 per iron atom of methæmoglobin

TABLE I

Acid methæmoglobin in trough $1 \cdot 3 \times 10^{-4}$ gm atom per l. Thickness 23 mm, length 150 mm.

Acid methæmoglobin in reaction vessels 5×10^{-4} gm atom Fe per l. Thickness of layer 6 mm.

No.	Methæmo-globin as Fe gm atom per l	H_2O_2 M	Reading of trough in mm	Percentage of free acid methæmo-globin	Percentage of H_2O_2-methæmo-globin	H_2O_2/Fe
1	5×10^{-4}	0	0	100	0	0·00
2	5×10^{-4}	$1 \cdot 7 \times 10^{-4}$	45	70	30	0·33
3	5×10^{-4}	$3 \cdot 4 \times 10^{-4}$	90	40	60	0·67
4	5×10^{-4}	$5 \cdot 0 \times 10^{-4}$	125	16	84	1·00
5	5×10^{-4}	$6 \cdot 7 \times 10^{-4}$	140	6	94	1·33
6	5×10^{-4}	$8 \cdot 3 \times 10^{-4}$	146	2	98	1·67

Oxyhæmoglobin is therefore not suitable for the control experiments and must be replaced by CO-hæmoglobin which is not so easily oxidized to methæmoglobin by H_2O_2 in presence of the same concentration of catalase.

Two kinds of manometric experiments have been used in this investigation. In the first series of experiments catalase was mixed with buffer, hæmoglobin, or methæmoglobin before the addition of H_2O_2; in the second series catalase was added soon after the introduction of H_2O_2 which had therefore time to react with methæmoglobin before it came into contact with the added catalase. These two series of experiments were carried out in two different types of flasks of differential manometers. For the experiments of the first series ordinary flat bottom flasks were used. In one of the typical experiments the left-hand flask received $3\cdot3$ cc of water while the right-hand flask received $2\cdot8$ cc of $2\cdot5 \times 10^{-3}$ methæmoglobin, $0\cdot2$ cc of catalase solution,* and $0\cdot02$ cc of phosphate buffer. $0\cdot28$ cc of $2\cdot5 \times 10^{-2}$ M H_2O_2 was put in a small cup provided with a platinum hook by means of which it can be suspended from the edge of the absorption tube (Keilin, 1929). For the control experiment the methæmoglobin of the right-hand flask is replaced by hæmoglobin of the same concentration. Both manometers are then filled with CO by a method previously described (1929). When the temperature of the flasks is equilibrated, the taps of the manometers are closed, the manometers are read, the cups are dislodged and H_2O_2 is thus mixed with the contents of the flasks. The manometers are read until they become stationary. In the flask containing CO-hæmoglobin most if not all of the H_2O_2 is decomposed by catalase, liberating molecular oxygen, the amount of which can be calculated from the manometric reading. In the flask containing methæmoglobin solution there is a partition of H_2O_2 between methæmoglobin and catalase and if the concentration of the latter is not too high most of the H_2O_2 added combines with methæmoglobin and thus escapes decomposition, while some of the H_2O_2 reacts with catalase liberating molecular oxygen.

The experiments of the second series were carried out in the Barcroft differential manometer with flat bottom flasks and provided with an external bulb connected with the flask by means of a carefully ground joint, fig. 2. The bulb is of the same shape as the side bulb in flasks previously described (Dixon and Keilin, 1934). It is provided with a gas outlet, and, when turned through 180°, delivers its contents into the

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* Catalase was prepared from fresh horse liver by the method described by Zeile and Hellström (1930) slightly modified.

flask. In a typical experiment of this series the left-hand flask receives 3·3 cc of buffer solution; the right-hand flask receives 2 cc of methæmoglobin, 7×10^{-3} gm atom Fe per l., 0·8 cc of acid phosphate M/4, 0·2 cc of 7×10^{-2} M. H_2O_2 in a suspended cup and 0·3 cc of strong catalase solution in the side bulb. In the control experiment the right-hand flask instead of methæmoglobin receives either hæmoglobin of the same concentration or simply phosphate buffer solution.
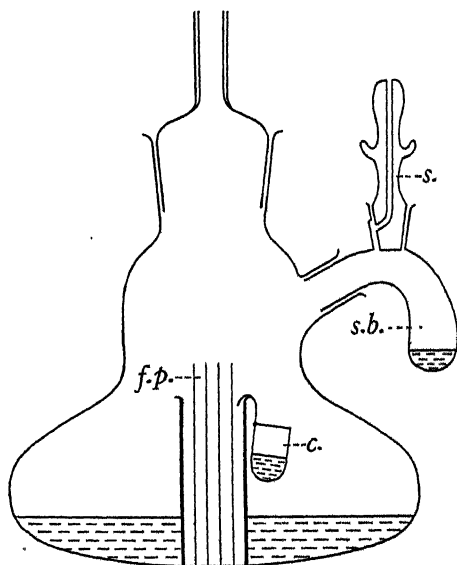
FIG. 2—Flask of a Barcroft differential manometer used in some of the experiments: c, cup for H_2O_2 solution; s.b., side bulb for catalase solution; s., stopper; filter paper (f.p.) in KOH tube and KOH are not necessary for the experiments described in this paper

When hæmoglobin is used as one of the control tests the flasks of all the manometers are filled with carbon monoxide. After equilibration of temperature on both sides of the apparatus, the taps are closed, the manometer is read, the cups are then dislodged and the H_2O_2 thoroughly mixed with the contents of the flasks. After shaking for about 2 minutes the side bulbs of the right flasks are turned so as to deliver the catalase into the flasks. The manometers are read until they become stationary and the amount of the oxygen liberated calculated from the manometric reading. The results of these experiments, which are summarized in Table II and figs. 3 and 4, clearly show that the amount of oxygen liberated from H_2O_2 by a catalase preparation in the presence of buffer represents from 91 to 99% of the theoretical amount. In the presence of CO-hæmoglobin the amount of oxygen liberated by catalase is from 84%

TABLE II

| CO-hæmoglobin | | Buffer | H$_2$O$_2$ | | Catalase | O$_2$ evolved | O$_2$ total in | Liberated O$_2$% of |
cc.	gm atom of Fe per l	cc	cc	M	cc	c.mm	H$_2$O$_2$	the total
2·5	3·2 × 10³	0·50	0·20	3·6 × 10⁻²	0·1	+80	80	100
2·8	2·5 × 10⁻³	0·22	0·28	2·5 × 10⁻²	—	+65	78	84
2·8	2·5 × 10⁻³	0·02	0·28	2·5 × 10⁻²	0·2	+66	78	85
2·8	2·5 × 10⁻³	0·02	0·28	5·0 × 10⁻²	0·2	+152	156	98
2·0	7·0 × 10⁻³	0·80	0·20	7·0 × 10⁻²	0·3*	+148	156	94
Methæmoglobin								
2·5	3·0 × 10⁻³	0·50	0·20	4·0 × 10⁻²	0·1	+15	88	17
2·0	4·7 × 10⁻³	1·10	0·20	5·3 × 10⁻²	—	+12	119	10
2·5	3·0 × 10⁻³	0·50	0·20	4·0 × 10⁻²	0·1	+13	88	15
2·5	3·0 × 10⁻³	0·30	0·20	3·6 × 10⁻²	0·3*	−7	80	—
2·8	2·5 × 10⁻³	0·02	0·28	2·5 × 10⁻²	0·2	0	78	0
2·0	7·0 × 10⁻³	0·80	0·20	7·0 × 10⁻²	0·3*	+19	156	12
2·0	7·0 × 10⁻³	0·80	0·20	7·0 × 10⁻²	0·3*	+31	156	20
—	—	3·00	0·20	4·0 × 10⁻²	0·1	+78	88	89
—	—	3·00	0·20	4·0 × 10⁻²	0·1	+84	88	95
—	—	2·80	0·20	3·6 × 10⁻²	0·3*	+89	80	111
—	—	3·00	0·20	3·6 × 10⁻²	0·1	+82	80	102
—	—	3·00	0·20	3·6 × 10⁻²	0·1	+89	80	111
—	—	2·82	0·28	5·0 × 10⁻²	0·2	+148	156	95
—	—	2·80	0·20	7·0 × 10⁻²	0·3*	+155	156	99

* Experiments of the second series in which catalase is delivered from the side bulbs after the additiol of H$_2$O$_2$.

to 100% of the theoretical amount, which shows that the H$_2$O$_2$ does not combine with hæmoglobin and is completely free and available for the reaction with catalase. On the other hand, in the presence of methæmoglobin, the concentration of which in gm atom of Fe is equal to the molar concentration of H$_2$O$_2$ (H$_2$O$_2$/Fe = 1), the amount of oxygen liberated by catalase varies from 0 to 20%, the remaining 100 to 80% being combined with methæmoglobin, is not available for the reaction with catalase. When the concentration of H$_2$O$_2$ is higher than that of methæmoglobin (H$_2$O$_2$/Fe = 2), one portion of it becomes available for reaction with catalase and liberates the corresponding amount of oxygen. For instance, 0·28 cc of 5 × 10⁻² M. H$_2$O$_2$ in presence of catalase and of CO-hæmoglobin liberates 152 c.mm of oxygen which is very near the theoretical amount, 156 c.mm. In the presence of catalase and methæmoglobin (H$_2$O$_2$/Fe = 1) the same amount of H$_2$O$_2$ liberates only 19 c.mm of oxygen, and in the presence of half the amount of methæmoglobin (H$_2$O$_2$/Fe = 2) it yields 93 c.mm which is not very far from what is

expected, namely, $156/2 + 19 = 97$ c.mm. The manometric experiments therefore corroborate our previous spectroscopic results, namely, that H_2O_2 combines with methæmoglobin in the proportion of one molecule of H_2O_2 to one atom of iron of methæmoglobin.

The Decomposition of H_2O_2-*methæmoglobin and the fate of* H_2O_2—We have mentioned previously that H_2O_2-methæmoglobin is very unstable and on standing even at room temperature soon reverts to the unbound
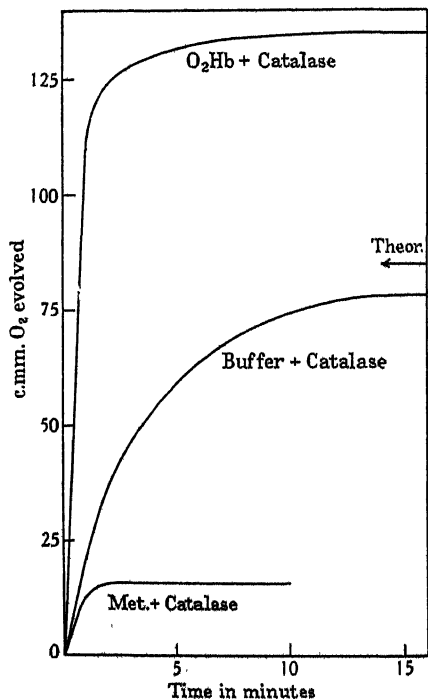
Fig. 3—Liberation of oxygen from H_2O_2 by catalase in presence of oxyhæmoglobin, buffer, and methæmoglobin

acid methæmoglobin. This decomposition proceeds very rapidly and can be easily followed spectroscopically. Thus a solution which contains 97% of H_2O_2-methæmoglobin and only 3% of acid methæmoglobin contains after 10 minutes standing 50% and after 30 minutes standing 70% of free acid methæmoglobin; after 60 to 70 minutes its concentration reaches the maximum of 87%, while the remaining 13% of methæmoglobin seems to have undergone destruction. It is important to mention here that the addition of catalase to H_2O_2-methæmoglobin does not accelerate the decomposition of this compound. This clearly shows that during the decomposition of H_2O_2-methæmoglobin and the liberation of acid

methæmoglobin, H_2O_2 is neither liberated and accumulated as such nor is it decomposed into oxygen and water. It seems to be used in a true oxidation reaction which is responsible for the partial destruction of methæmoglobin. The decomposition of H_2O_2-methæmoglobin is, on the other hand, greatly accelerated by the addition of suitable easily oxidizable substances, such as p-phenylenediamine, benzidine, or traces of $Na_2S_2O_4$.
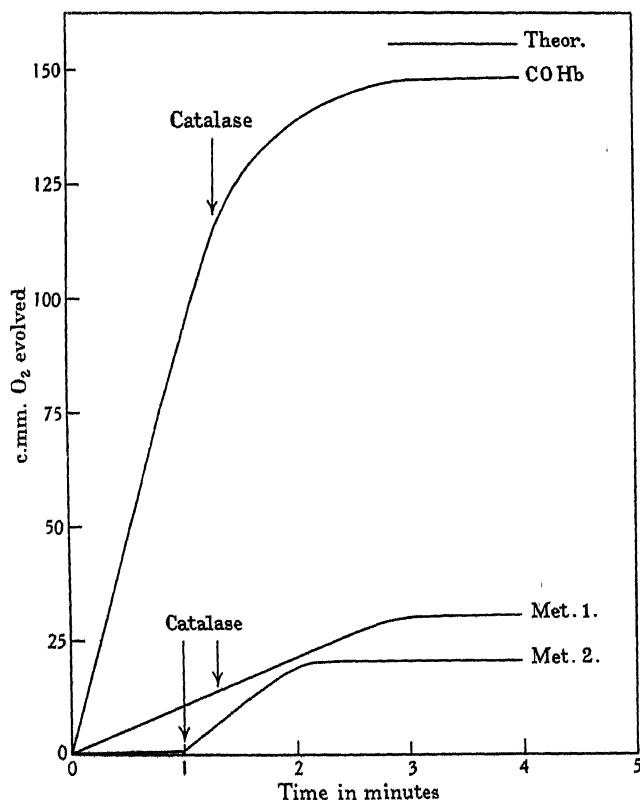
FIG. 4—Liberation of oxygen by catalase in presence of CO-hæmoglobin and two solutions of methæmoglobin. CO-hæmoglobin and methæmoglobin 1 have not been completely purified from catalase

In these cases methæmoglobin acts as a peroxidase and the H_2O_2 combined with it is rapidly used in oxidation of these compounds, leaving free acid methæmoglobin. Moreover, it is well known that KCN forms a fairly stable compound with methæmoglobin; when KCN is added in excess to H_2O_2-methæmoglobin it gradually replaces H_2O_2, forming cyanmethæmoglobin. This reaction is also accelerated by the addition of the above-mentioned oxidizable substances.

That the H_2O_2 does not yield molecular oxygen during decomposition of H_2O_2-methæmoglobin is corroborated also by the manometric experiments.

In these experiments the spectroscopic examination of the content of the flask immediately after addition of H_2O_2 shows only the absorption bands of H_2O_2-methæmoglobin. On the other hand, 30 to 45 minutes later most, if not all, of the methæmoglobin is seen to be in the free state. The manometric reading does not reveal, however, any liberation of oxygen from the H_2O_2 which was previously combined with methæmoglobin. The oxygen is not liberated even if fresh catalase is added. H_2O_2 disappears, therefore, from solution without being decomposed into molecular oxygen and water. It seems to be used as we have previously suggested in a true oxidation reaction as is promoted by peroxidase.

These observations show how dangerous it is to ascribe a catalatic property to a substance or to a system when relying only on the disappearance of H_2O_2 added to the system. Unfortunately during the last few years the catalatic properties of several compounds were determined (and estimated) only by titration of the residual unmodified H_2O_2 with a standard solution of $KMnO_4$. We see now that such methods can be safely used only where the mechanism of disappearance of H_2O_2 has been previously ascertained.

COMBINATION OF METHÆMOGLOBIN WITH ETHYL HYDROPEROXIDE (C_2H_5OOH)

Ethyl hydroperoxide was prepared by the method of Bayer and Villiger (1901) from diethylsulphate and perhydrol. In view of the explosive nature of this peroxide only a small quantity was prepared and purified. From 30 gm of Merck's perhydrol we have obtained 3·5 gm of 76·5% ethyl hydroperoxide. The concentration of the solution was determined iodometrically by titration with standard thiosulphate. A sample of 0·2 M solution of ethyl hydroperoxide after 2 weeks standing in an ice chest was tested with titanium and revealed only 5×10^{-4} M free hydrogen peroxide. Such a negligible concentration of H_2O_2 could not affect in the least the results of our experiments which will be presently described.

Ethyl hydroperoxide, not being affected by catalase, can be used in the experiments with methæmoglobin rich in catalase prepared directly from a solution of unpurified hæmoglobin. Ethyl hydroperoxide reacts with methæmoglobin in the same way as H_2O_2, turning the brown solution

into a red one which shows two diffuse absorption bands at 589 mμ and 545 mμ.

The minimum amount of C_2H_5OOH required for the formation of ethyl hydroperoxide-methæmoglobin was determined spectroscopically using the same method as for H_2O_2.

Table III, which summarizes these experiments, clearly shows that the formation of ethyl hydroperoxide-methæmoglobin requires one molecule of peroxide for each atom of iron of methæmoglobin.

TABLE III

Acid methæmoglobin in trough $1 \cdot 33 \times 10^{-4}$ gm atom of Fe per l.; thickness 23 mm; length 150 mm.

Acid methæmoglobin in reaction vessels 5×10^{-4} gm atom of Fe per l.; thickness of layers 6 mm.

No.	Methæmo-globin as gm atom Fe per l.	C_2H_5OOH M	Reading of trough in mm	Percentage of free methæmo-globin	Percentage of C_2H_5OOH methæmo-globin	$\dfrac{C_2H_5OOH}{Fe}$
1	5×10^{-4}	0	0	100	0	$0 \cdot 00$
2	5×10^{-4}	$1 \cdot 7 \times 10^{-4}$	40	73	27	$0 \cdot 33$
3	5×10^{-4}	$3 \cdot 3 \times 10^{-4}$	85	43	57	$0 \cdot 67$
4	5×10^{-4}	$5 \cdot 0 \times 10^{-4}$	122	18	82	$1 \cdot 00$
5	5×10^{-4}	$6 \cdot 7 \times 10^{-4}$	135	8	92	$1 \cdot 33$
6	5×10^{-4}	$8 \cdot 3 \times 10^{-4}$	145	3	97	$1 \cdot 67$

This compound is also unstable liberating methæmoglobin on standing. Ethyl hydroperoxide, during this process of decomposition, is used up in a true oxidation reaction. The rate of this decomposition, as with H_2O_2-methæmoglobin, is greatly accelerated by the addition of oxidizable substances such as benzidine or para-phenylendiamine.

OXIDATION OF OXYHÆMOGLOBIN BY PEROXIDES

According to Haurowitz (1924, pp. 17, 27) H_2O_2 does not oxidize hæmoglobin to methæmoglobin, although this oxidation is produced by a great variety of reagents such as potassium ferricyanide, potassium permanganate, quinone, hydroxylamine, nitrites, formaldehyde, glycerin, etc. We think, however, that his failure to oxidize hæmoglobin with H_2O_2 was undoubtedly due to a high concentration of catalase in his solutions of hæmoglobin. In fact, while H_2O_2 has no effect on crude oxyhæmoglobin, it rapidly oxidizes the purified hæmoglobin recrystal-

ized several times. Moreover, this oxidation can be easily demonstrated even in the preparation not purified by crystallization, if only the activity of catalase is abolished by the addition of small concentrations of KCN or of NaN_3. Ethyl hydroperoxide, on the other hand, when added to oxyhæmoglobin even in the presence of active catalase rapidly oxidizes it to methæmoglobin, which combines with the remaining peroxide forming ethyl hydroperoxide-methæmoglobin compound. These experiments explain also the results recently obtained by Abragam and Magat (1934) on the variation in the appearance of hæmoglobin in fowls produced by injections with " lecithin-perhydrite " compound. By injecting the fowls with a mixture of " stabilized colloidal lecithine " and perhydrite (compound of H_2O_2 and urea) they have noticed a rapid darkening of the comb and of the blood. This darkening gradually subsides and 48 hours after injectiont he blood re-acquires its normal colour. The changes in the colour and the absorption curves given by these authors clearly indicate that their injections have partly transformed the hæmoglobin into methæmoglobin which has partly combined with the free peroxide. The solution of blood which they have examined spectroscopically contained therefore a mixture of : (1) oxyhæmoglobin, (2) methæmoglobin (alkaline and acid), and (3) H_2O_2-methæmoglobin. Their conclusion that " It is thus possible to introduce oxygen into the blood intravenously " is incomprehensible. The fraction of H_2O_2 used in their injection which was not decomposed by catalase was used up in the following way:

(1) In the oxidation of the divalent iron of reduced hæmoglobin to the trivalent iron of methæmoglobin; (2) in a similar oxidation of oxyhæmoglobin to methæmoglobin; (3) in the formation of the H_2O_2-methæmoglobin compound and the ultimate decomposition of H_2O_2 in a true oxidation reaction without liberation of molecular oxygen (cf, p. 10). In their experiments the oxygen capacity of the blood was therefore not increased but diminished by the amount equivalent to the hæmoglobin which has undergone oxidation to methæmoglobin.

SUMMARY AND CONCLUSIONS

Methæmoglobin containing little or no catalase was prepared from crystalline horse hæmoglobin.

Solutions of acid methæmoglobin, on addition of H_2O_2, turn from brown to red, and the characteristic absorption spectrum of acid methæmoglobin is replaced by two diffuse bands at 589 mμ and 545 mμ.

This change of colour and absorption spectrum is due to the formation of a real compound between H_2O_2 and methæmoglobin.

The H_2O_2-methæmoglobin is analogous to the compounds formed between methæmoglobin and KCN, KF, H_2S, and NaN_3, from all of which unmodified hæmoglobin can be recovered.

By spectroscopic and manometric methods it was found that the formation of H_2O_2-methæmoglobin requires one molecule of the H_2O_2 per atom of iron of methæmoglobin.

Ethyl hydroperoxide (C_2H_5OOH) combines with methæmoglobin forming a compound similar in every respect to H_2O_2-methæmoglobin.

The formation of ethyl hydroperoxide-methæmoglobin also requires one molecule of ethyl hydroperoxide per atom of iron of methæmoglobin.

Both peroxides (H_2O_2 and C_2H_5OOH) oxidize hæmoglobin to methæmoglobin. While ethyl hydroperoxide does so even in the presence of catalase, H_2O_2 acts only on hæmoglobin more or less free from catalase or when the activity of the latter is inhibited by KCN or NaN_3.

Both peroxide-methæmoglobin compounds are unstable and on standing even at room temperature revert to acid methæmoglobin.

During this decomposition, H_2O_2 is not split into molecular oxygen and water and both peroxides seem to be utilized in a true oxidation reaction such as is promoted by peroxidase.

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The Effects of Prolonged Œstriol Administration upon the Sex Skin of *Macaca mulatta*

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[Plates 1-4.]

Introduction

The work of Collings (1926), Allen (1927, 1928), Morrell (1930), and Parkes and Zuckerman (1931) on rhesus monkeys has established the fact that the brilliant coloration of the skin about the genitalia and the face, loosely referred to as the sex skin, and seen in both the female and male of this species, can be called forth by the administration of œstrous-producing hormone. Parkes and Zuckerman could not produce it in the case of one castrate male rhesus and a castrate female bonnet monkey, *M. radiata,* but they obtained in an ovariectomized baboon the full swelling seen during the follicular phase, and Dohrn, Hohlweg and Schoeller (1932, 1933) working with both male and female baboons and employing crystalline "progynon," were uniformly successful in eliciting a reaction, which in the male specimens of this species consisted of a remarkable œdema of the genitalia. In the present paper we should like to report our own experiments concerning this particular skin reaction to œstriol.

Material and Technique

Eight macaques, including one immature and four adolescent animals, were studied. Of the total, five were normal animals, four of these being males. The remaining three comprised one castrate male and two hypophysectomized-castrate females.

Since, as is well known, the sex coloration is influenced considerably by environmental conditions and by the state of the animal's general health, it seems pertinent to add that the group here studied was housed indoors throughout the experiments, in individual cages allowing little room for activity. All animals, however, with the exception of one of the

hypophysectomized pair, remained in excellent condition, gaining weight on a mixed " purina " and fresh food diet similar to that employed by Corner (1933).

The injections were continued over periods varying from 12 to 110 days. Crystalline œstriol was used, and was given subcutaneously in 10% alcoholic solution in dosages of 200 γ thrice daily in the beginning; later 500 γ was given once daily in oil.

<div align="center">OBSERVATIONS</div>

The initial response to the above dosages of œstriol is seen within 48 hours, and begins with erection and reddening of the nipples, and a dusky or cyanotic œdema of the anus and adjacent base of the tail. Within another day or two the skin of the genital region begins to redden and swell, until—after 8 to 10 days of injections—the entire area overlying the pubis, the inner sides of the thighs, the perineum and proximal third of the tail is raised in a localized œdematous swelling having a pinkish hue. The swelling is sharply demarcated over the pubis and along the inner thighs from the adjacent normal skin of the abdomen and legs, but elsewhere, as over the buttocks and along the tail, merges gradually with the uninvolved surrounding parts. The intensity of the swelling at its height is such as to render the overlying epidermis tense and glistening and to elevate the perineal skin flush with the usually protuberant ischial callosities. Contrasted to the œdema which later occurs elsewhere, this swelling pits upon pressure. In the female the labia majora and nymphæ are swollen to several times normal size and a moderate mucous vaginal discharge appears. In the male the process involves the scrotum and the penile skin, but the cavernous shaft of the penis remains apparently little, if at all, changed. Both immature and mature males develop huge, pendant, tense scrotal enlargements. In adults the swelling of the prepuce and penile sheath may be such as actually to embarrass the free flow of urine. In immature animals, there is no palpable enlargement of the testes nor any tendency to descent of the same into the pendant scrotal sac.

If the œstriol injections are continued, these phenomena remain at the above maximum for a period varying from 3 to 10 days. At the height of this stage the hairless portion of the face is brilliantly flushed. The animal is very restless and more than usually difficult to catch and handle.

With further administration of œstriol the above phase, which in many respects constitutes a mere duplication of the physiological swelling

of the sex skin of rhesus, and which we shall for convenience designate as the initial phase, gradually subsides, giving place to a secondary response which is remarkable in many respects. The subsidence of the initial phase occupies from 10 to 40 days, depending apparently upon the maturity of the animal. In some animals, however, this is never altogether complete, and a residium of coloration and skin puckering in the genital area persists as long as treatment is continued. The major feature of the fading period is the disappearance of the initial œdema first from the anus and later from the thighs and genitals.

Irrespective of the speed of subsidence of the initial phase, continuously treated animals begin, between 20 and 25 days, to develop a secondary and for the most part pallid or colourless œdema of entirely different type and distribution from that of the initial reaction. Beginning as a series of localized bulbous and deeply indented swellings along the lines of the sartorii from groin to knee, it next appears over the lumbar region, spreading thence gradually upward to involve the entire dorsal and hairy portion of the trunk. Simultaneously the skin upon the supraorbital ridge becomes swollen in a continuous line from the malar eminences across the orbits and root of the nose. An œdematous fold appears on the lower abdomen, extending from symphysis to umbilicus. Finally, and usually within 10 to 15 days, the swellings spread downward along the extensor folds of the legs and arms, the picture being complete when the ankles and wrists present œdematous cuffs.

The œdema of this stage does not yield to pressure and appears to involve the integument only, the latter being thrown up into a thickened, armour-like plate riding easily over the subcutaneous tissues, and everywhere deeply cut by an irregular pattern of grooves with intervening tensely swollen ridges. The predilection of the œdema for the hairy regions, and the marked distortion of the hair follicles, throws the fur into a ruffled and erect condition. The affected areas, moreover, appear to be relatively insensitive.

Unlike the first phase, this secondary reaction persists at maximum intensity throughout the injection period—which in one animal was extended as long as 110 days. When the hormone is ultimately discontinued, it takes 8 to 10 days before this reaction begins to fade, whereupon about 30 more days are required for all traces of it to disappear. Simultaneously with the first observable subsidence of œdema female animals begin to bleed from the vagina.

If the treatment is discontinued at the peak of the first stage, that is when the swelling of the genital area reaches its full development, the reaction fades immediately and the second stage of extragenital œdema
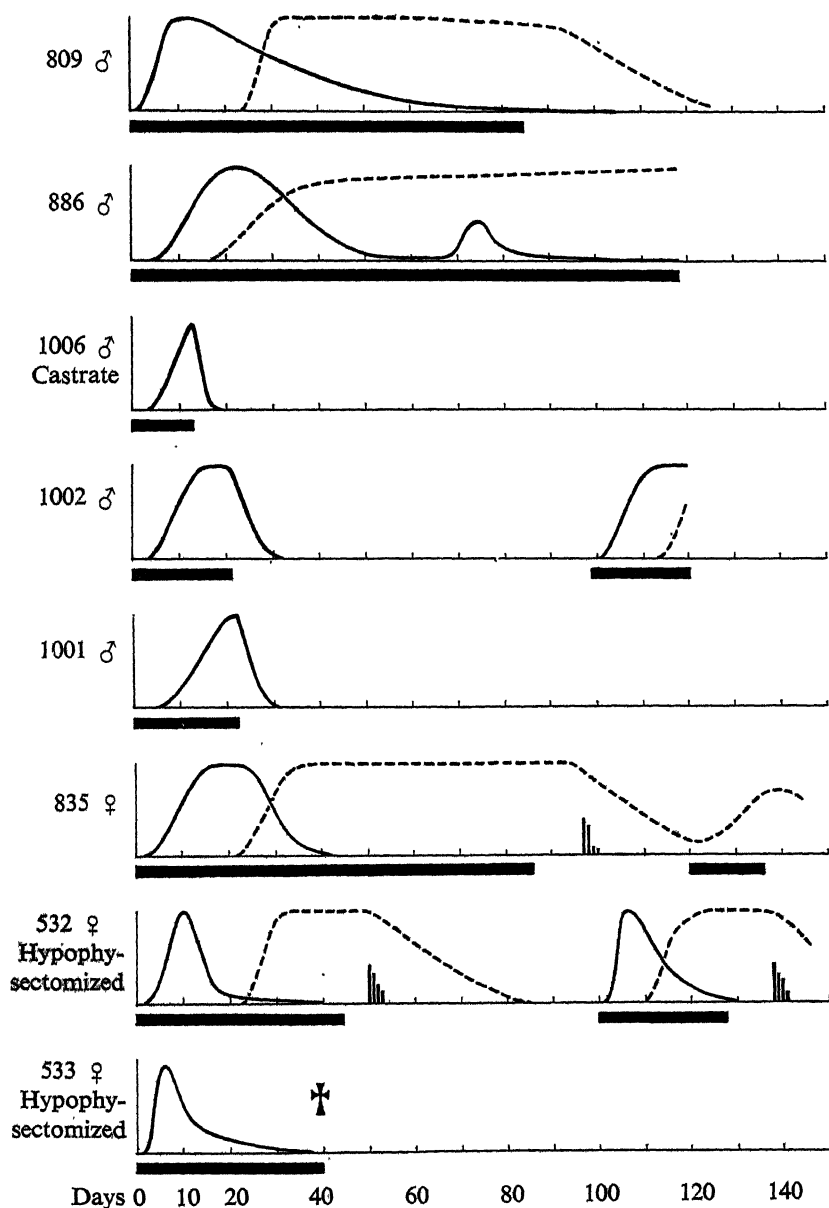
FIG. 1—Diagram showing the development of the sex skin in our experimental
animals. — Genital œdema; – – Extra-genital œdema; ▬ œstriol; ||| Vaginal
bleeding

does not appear. In those cases in which the treatment is discontinued during the secondary (extragenital) reaction the subsidence of the œdema is markedly less rapid.   .

Since the skin of the genital area shows no response to œstriol during the later stages of treatment, it was of interest to establish whether it remains unresponsive after injections are discontinued for some time. For this purpose three animals were subjected to a second series of injections after varying intervals of rest. In these experiments no genital swelling was obtained if treatment was reinstituted before full subsidence of the œdema from the preceding period of treatment; the response manifested itself immediately in the extragenital areas. If the interval was longer, the usual genital reaction preceded the extragenital manifestations.

Castration, both in the male and in the two females available for study, was apparently without influence on either the speed or intensity of the various phases of response to œstriol.

In the two hypophysectomized castrates studied, there was neither qualitative nor quantitative difference in response as compared with that of normal animals, but the initial reaction was considerably shortened. One of these animals died of intercurrent disease before complete observations could be made; the completeness of the pituitary removal could therefore be verified post-mortem. The other animal, still in the colony had a grossly normal menstruation beginning 7 days after œstriol was discontinued, the animal at the time exhibiting the complete phenomena of the secondary reaction.

HISTOLOGY

Histologically the œdematous areas are characterized by the looser texture of the connective tissue fibres in the corium. There are many large polygonal fibroblasts with vacuolated cytoplasm in this region and the smaller blood vessels of the cutis are surrounded by sheaths of small round cells. Numerous cells are also found in the subepidermal layers of the skin. These changes are not unlike those seen in rats after the administration of parathyroid hormone. It is noteworthy that the epidermis itself is also markedly thicker in the œdematous region than elsewhere.

SUMMARY

The reaction of the skin to chronic treatment with crystalline œstriol has been studied in male and female macacus monkeys under various experimental conditions. Histologically this skin reaction is character-

Fig. 1
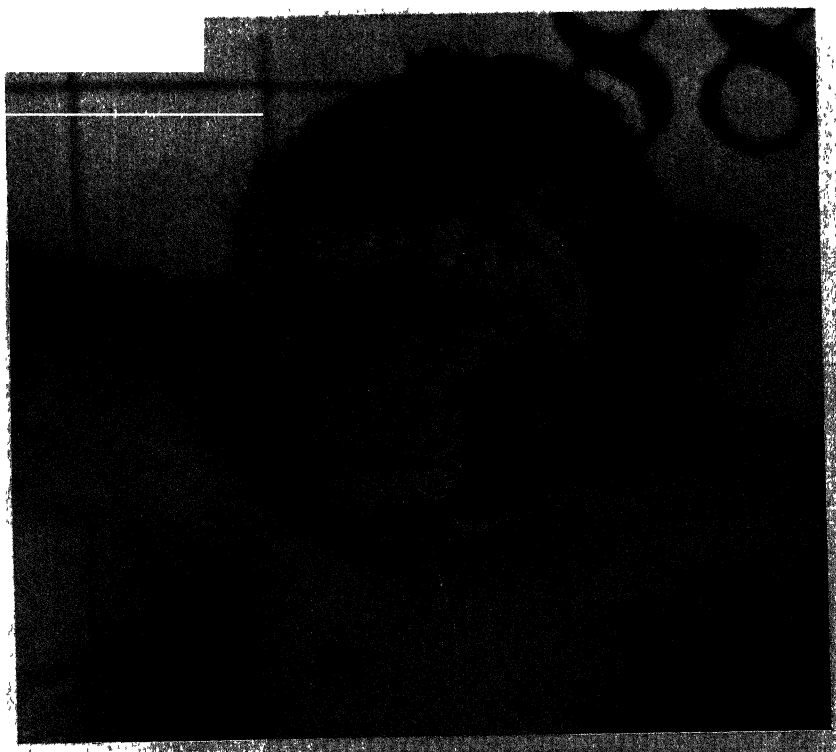
Fig. 2

Fig. 3

Fig. 4
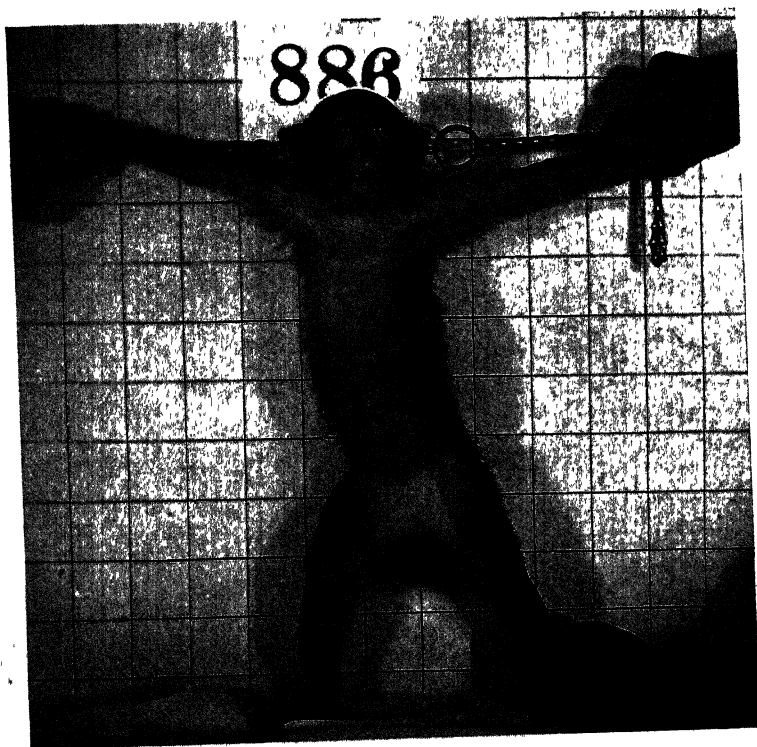
FIG. 5
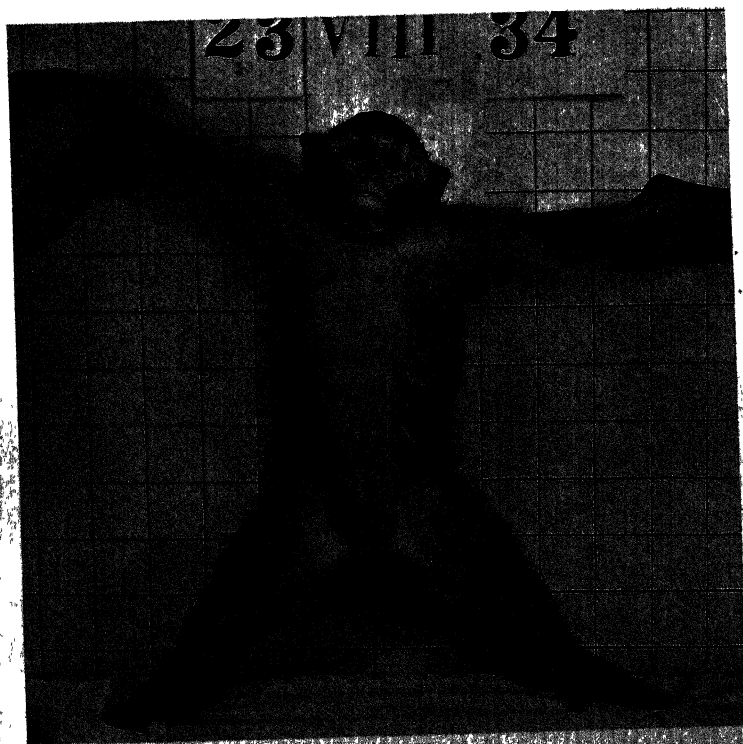
FIG. 6
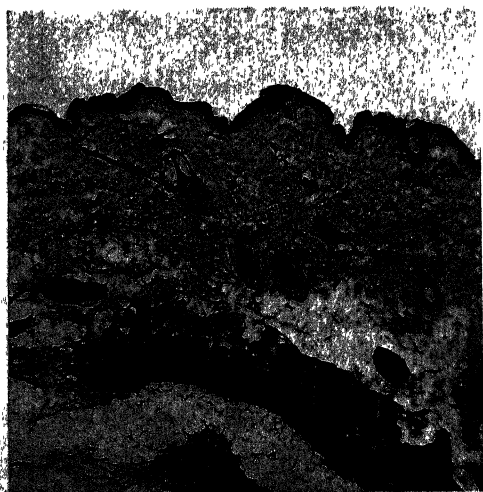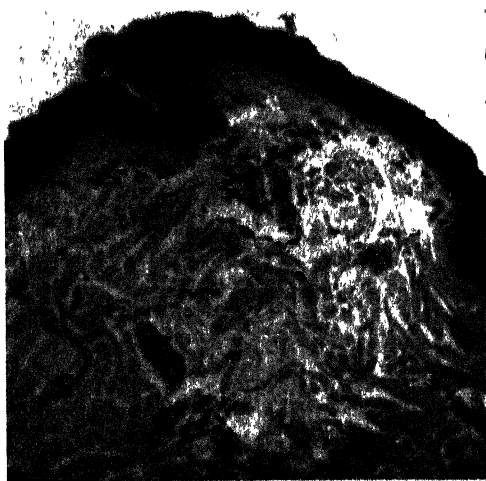
Fig. 7

Fig. 7B
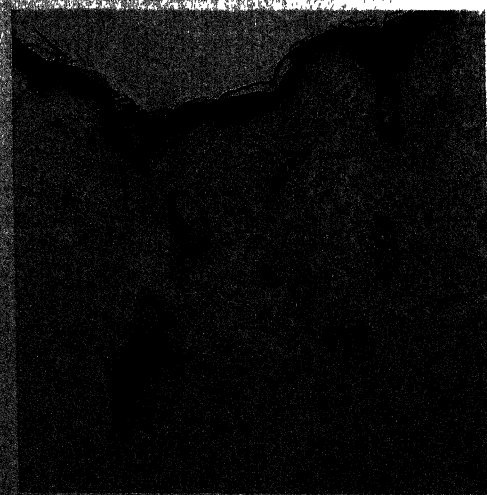
Fig. 8

Fig. 8B

ized by definite changes in the cells and the intercellular tissue of the corium, and by marked thickening of the epidermis.

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DESCRIPTION OF PLATES

PLATE 1

FIG. 1—Normal macacus monkey.
FIG. 2—Facial sex skin development in œstriol treated animal.

PLATE 2

FIG. 3—Genital sex skin development in œstriol treated male animal.
FIG. 4—Numerous œdematous ridges on the back and along the thighs during the
    later stages of œstriol treatment (secondary reaction).

PLATE 3

FIG. 5—Genital œdema during first stage of œstriol treatment (primary reaction).
FIG. 6—Extragenital œdema during second stage of œstriol treatment. Note that
    facial œdema, which begins during the first stage, is still present at this period,
    while the genital swelling has already subsided.

PLATE 4

FIG. 7—Histological section through the skin of normal monkey. Low magnifica-
    tion.
FIG. 7b—Same slide as shown in fig. 7, under high magnification.
FIG. 8—Histological section through the skin in the sexual area of a male
    during the primary reaction stage. Same magnification as fig. 7. Note the
    increase in thickness of the epidermis, the loose texture of the corium, the con-
    nective fibres of which are quite irregularly distributed.
FIG. 8b—Part of slide shown in fig. 8. Same magnification as fig. 7b. Note the
    increase in size of the connective tissue cells.

The Effects of X-Radiation on Chromosomes in the Microspores of *Trillium erectum* Linn.

By C. Leonard Huskins and A. W. S. Hunter, Department of Genetics, McGill University, Montreal

Previous studies of the effects of X-radiation upon somatic chromosomes have been subject in varying degree to three limitations. First, they have usually been upon tissues in which it was not possible to determine accurately the stage of division in any particular cell at the time of irradiation. Secondly, it has generally not been possible to determine directly or definitely the number of nuclear generations between the time of irradiation and fixation of any given cell. Thirdly, the observations have been mainly upon chromosomes so stained that their component chromatids have not been clearly distinguishable.

In the present study these limitations have either been very greatly minimized or eliminated. The material has the further advantage that the chromosomes of the normal complement are individually distinguishable, though fragmentation and translocation may make their identification difficult or sometimes impossible after irradiation.

Material and Methods

X-ray facilities were very kindly provided by Dr. E. C. Brooks, of the Ross X-ray Department, Royal Victoria Hospital, Montreal.

Thirty-five corms of *Trillium erectum* L. were irradiated on December 22, 1933, and January 9, 1934. The treatments were: 90 kV, 5 mA, 30 cm unscreened, for 2, 7, or 14 minutes. The 7- and 14-minute treatments proved to be excessive. Immediately after treatment the corms were planted in boxes, one of the anthers was removed from each bud, smears were prepared, fixed in S_2 (see Huskins and Smith, 1935, for formula) and stained with crystal violet. The six anthers in each *Trillium* bud are usually very uniform in their stages of meiosis or pollen-grain mitosis. The remaining anthers were taken at 1–3 day intervals after treatment. They were removed through a small incision in the side of each bud, a glass vial being placed over it to prevent drying-out. Development appeared to be normal for about 10 days after this treatment; moulds usually destroyed buds left longer under the vials.

The observations here reported in any detail are all from buds irradiated in the homœotypic telophase or early pollen-tetrad resting stage and examined in the first pollen-grain division. The excessive 7- and 14-minute irradiations damaged most of the nuclei so badly that detailed analysis of their effects was impossible, and unfortunately for some purposes most of the buds which were at earlier meiotic stages were included in the excessive treatments.

OBSERVATIONS

In preparations made immediately after irradiation no morphological effects were observed. In all specimens irradiation checked development. No divisions were found in any preparations made between the first and sixth day after treatment. In buds given the 7- or 14-minute treatment, most of the pollen mother-cells degenerated without further division. Multinucleate cells were formed with high frequency in one plant which had been irradiated for 2 minutes during the heterotypic meiotic division.
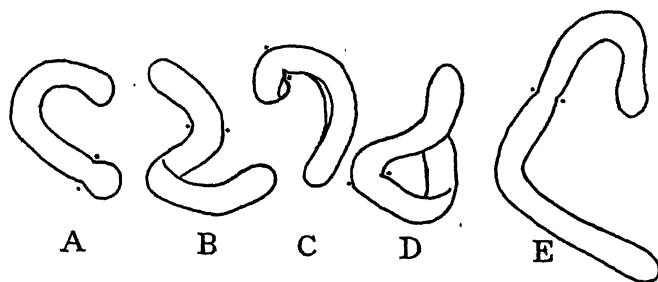
FIG. 1

The normal haploid somatic chromosome complement is illustrated in outline, without reference to the internal chromonema structure, in fig. 1, which is drawn from a pollen grain first mitotic metaphase fixed immediately after irradiation. The five chromosomes are designated A–E in conformity with the usage of Huskins and Smith (1935).

Both "chromosome" and "chromatid" breaks were found in a number of nuclei irradiated in the early pollen-tetrad "resting stage." "Chromosome" breaks, *i.e.*, breaks affecting both chromatids at one locus are shown in fig. 2, drawn from a single nucleus at prometaphase 7 days after irradiation for 2 minutes. This is, as in all cases here described, definitely the first nuclear generation after irradiation. Figs. 2 *b*, 2 *c*, and 2 *d* are apparently chromosomes B, C, and D, unaltered by the treatment. Chromosome breaks are shown in figs. 2 *a* and 2 *e*. In addition

there has probably been reciprocal interchange between chromosome
A and E to produce these configurations, since fig. 2 e has two attachment
constrictions, while none is evident in fig. 2 a.

Chromatid breaks in the same material are shown in fig. 3. There is
little doubt that figs. 3 a, 3 d, and 3 e represent chromosomes A, D, and E
respectively. Chromosome fragmentation is illustrated in fig. 3 b since

a   b   c   d   e
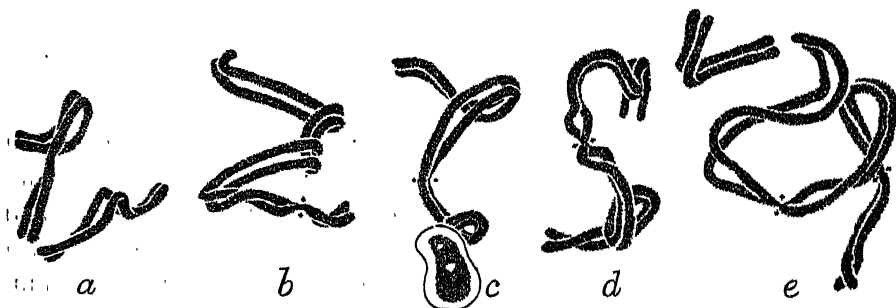
FIG. 2

it is shorter than any normal chromosome. There is both chromosome
fragmentation and chromosome translocation in fig. 3 c, since its left
arm is broken off and its right arm is longer than any normal chromo-
some arm. In figs. 3 a, 3 b, 3 d, and 3 e there are clear chromatid breaks;
fig. 3 d shows a chromosome break also. The relationship of parts
indicated by the dotted lines in fig. 3 d is readily seen in the preparation,

a   b   c   d   e
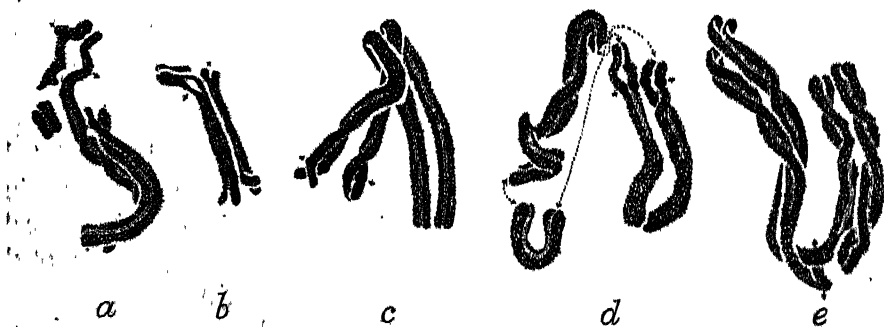
FIG. 3

but the chromosomes overlap so much in different focal planes that
drawings of them in situ are confusing. They have therefore, as in all
other cases, been drawn separately.

The nucleus represented in fig. 3 was at the metaphase and both of the
chromatids in each chromosome are very clearly split " in readiness for "
the anaphase of the succeeding division. In figs. 3 b, 3 c, and 3 d the chro-

matids are very clearly split at the " spindle fibre attachment " (indicated by arrows) as well as elsewhere along their length. The somatic chromosomes of *Trillium* pollen grains are very clearly four-partite at metaphase or the beginning of the anaphase in both X-rayed and untreated material.

The chromatids are not visibly split in fig. 2 which represents an earlier prometaphase or late prophase stage—the chromosomes are longer, they lack orientation and the nucleolus is still present.
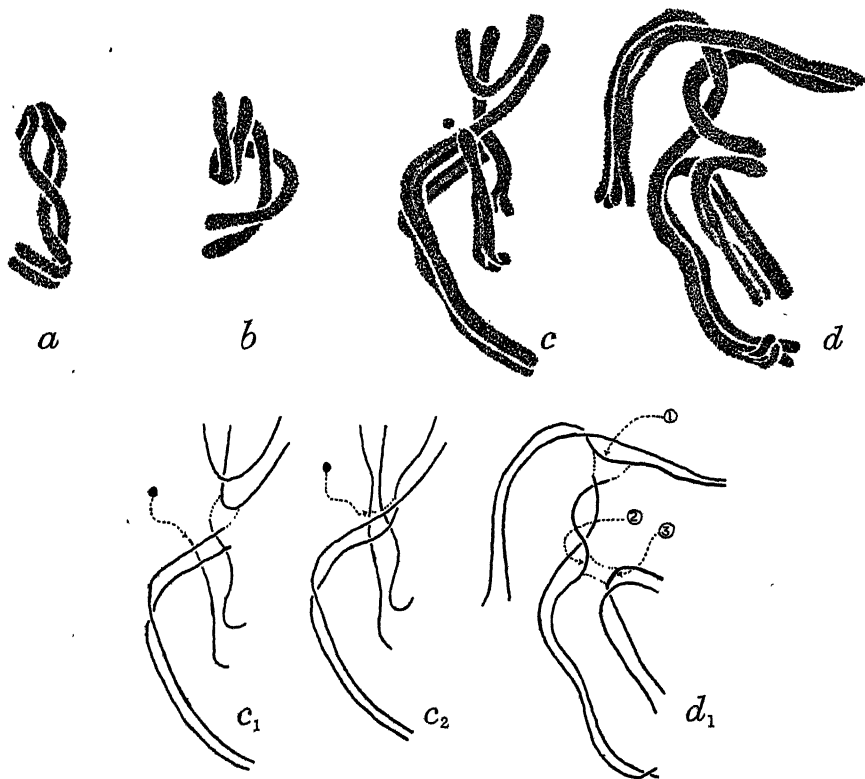
FIG. 4

Fig. 4 represents a single metaphase nucleus in which extensive changes have been produced. The chromosomes shown in figs. 4 *a* and 4 *b* are possibly A and B of the normal complement. The changes shown in fig. 4 *c* include a lateral translocation which may have involved a broken chromatid joining up with two chromatids from different chromosomes or chromosome fragments. Two possible interpretations of this are shown in diagrams 4 c_1 and 4 c_2. The dotted lines indicate the manner in which the chromatids are presumed to have rejoined. A single " trabant " occurs in this nucleus attached to a fragmented chromatid. There are

no trabants in untreated nuclei. The changes illustrated in fig. 4 *d* are interpreted in diagram 4 d_1. They are believed to involve the simple lateral attachment at point 1 of a chromosome which has itself undergone interchange with a third chromosome. This interpretation involves chromatid breaks having occurred at points 2 and 3.

Simpler lateral translocations are illustrated in figs. 5, 6, and 7. They are from different prometaphase nuclei. Figs. 5 and 7 are self-explanatory. Fig. 6 involves an unusual feature in having two single chromatids widely separated.

Chromatid breaks are shown in figs. 8 and 9; in the former the boundary of the de-stained matrix is illustrated. Since it seemed possible that some
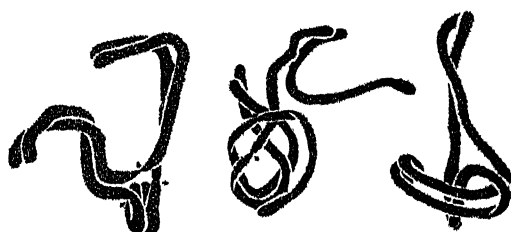
FIG. 5   FIG. 6   FIG. 7
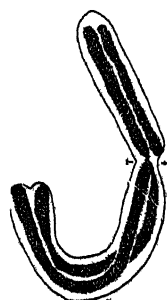
FIG. 8   *a*   *b*   FIG. 10
          FIG. 9

at least of the " constrictions " reported by other workers to be produced by irradiation might really be chromatid breaks, drawings were made from one slide which was prepared in the more customary manner, in which the matrix is deeply stained, and it was then bleached and re-stained to show its internal structure. The results are shown in figs. 9 *a* and 9 *b*. The two " constrictions " are clearly chromatid breaks.

A ring chromosome is shown in fig. 10. It is from a first pollen-grain metaphase nucleus. Unfortunately the remaining chromosomes of this nucleus are so badly fragmented that it is impossible to say which original chromosome is here involved. The intertwining of chromatids here evident may have significance for theories of chromosome splitting and " spiralization."

A pollen grain prometaphase or metaphase nucleus fixed on the 11th day after irradiation for 7 minutes at the tetrad stage is shown in fig. 11 to illustrate how badly the chromosomes may be damaged and yet still begin to undergo mitosis.

DISCUSSION

Translocations and breaks observed by previous workers have been almost exclusively " chromosome " translocations or breaks, *i.e.*, they have involved both chromatids at one locus. Lewitsky and Araratian (1931, fig. 27) found one example of a break occurring in a single chromatid in irradiated *Crepis capillaris*. Mather and Stone (1933) state that they found no chromatid breaks or trans-locations, and they argue that Lewitsky and Araratian's example " was probably due to a slight upset in the time of pro-duction of abnormalities or of the splitting of the chromosomes." They deduce from their observations that chromosomes split during the resting stage, not at the pre-ceding division, as many recent authors maintain. This issue will be referred to again. Mather and Stone's fig. 22 shows the lateral translocation of an apparently single trabant, but they evidently presume that it is really double. They state further that the attachment of a clearly double trabant in their fig. 23 shows that " the translocation must have occurred before

FIG. 11

the split of the chromosomes as it is exceedingly improbable that two equal translocations should occur laterally at the same corresponding places on sister chromatids." The fact that the nucleus in which the double trabant occurs was fixed 5 days after irradiation, and that they found divisions begin-ning 2 days after irradiation leaves open the *possibility* that this was the second division after treatment. If that were so their argument lacks significance, as does also their conclusion that this figure *proves* trabants to be merely small pieces of chromosome, since lateral chromatid translocations of a trabant normally situated terminally on another chromosome could give second cell generation nuclei with two terminal trabants on one chromosome and two lateral ones on another. Trabants which must have originated *de novo*, since none are present in normal *Trillium* nuclei,
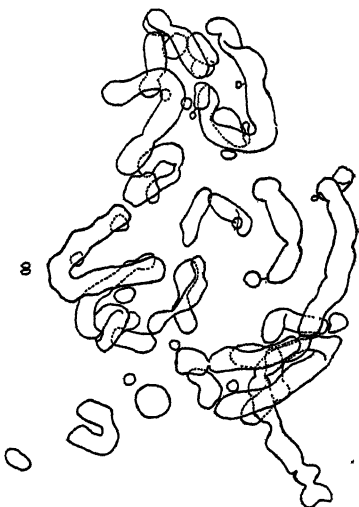
were found in many cells in the present material, cf. figs. 4 and 11.   The problem of the nature of trabants is not, however, solved thereby as Mather and Stone would maintain.   These "trabants" are clearly only small pieces of chromosome, but study of their behaviour in subsequent cell divisions would be necessary to prove that they are real trabants having the special properties claimed for trabants by numerous authors (cf. Sharp, 1934).

It has been very generally observed that the effects of irradiation become apparent chiefly in the anaphase of the first division occurring after treatment (Goodspeed, 1929; Stone, 1933; Helwig, 1933). Strangeways and Hopwood (1927) conclude that there is a stage immediately prior to the visible prophase, during which X-rays are particularly effective. Goodspeed (1929) has suggested that X-rays may disrupt the molecular arrangements within the chromosome causing a degree of molecular instability "a disturbance which will express itself in fragmentation only when the chromosome comes under the influence of an energized cytoplasm."   Hanson and Heys (1929) found that radium and X-rays have much less effect genetically upon early than upon late male germ cells of *Drosophila melanogaster*.   Muller (1930) decided that there is little genetic evidence of a delayed induction of mutations.   He concludes that the tendency for a mutation to appear in only one half of a fly derived from treated sperm indicates not that the mutation is delayed to the two nuclear stage of cleavage, but rather that the chromosomes may already be split in the mature spermatozoon.   Our results very clearly support Muller's interpretation.   They show that chromatid breaks will not be obvious before anaphase in chromosomes which are not stained to show internal structure, and that they may, owing to the matrix or sheath holding parts together, not be seen until the next division (cf. also Metz, 1934).   On the other hand, chromosome breaks should be, and are, obvious before the first mitotic metaphase following irradiation. The results of Goodspeed (1929) and Stone (1933) can be interpreted on this basis, though the possibility of a molecular or physiological disruption which they favour as an inclusive explanation is, of course, not ruled out as a partial one.

Lateral translocations in irradiated *Drosophila*, *Nicotiana*, and *Crocus* have been described by Painter and Muller (1929), Muller and Painter (1929), Goodspeed and Avery (1930) and Mather and Stone (1933), and by Darlington (1929) and Levan (1932) in untreated *Tradescantia* and *Allium*.   Certain genetic results in *Drosophila* (Bridges, 1923; Hamlett, 1926) have been interpreted on the assumption of a lateral translocation which gave a more or less permanently branched

chromosome. It was not observed cytologically. Our observations of lateral translocations attached to only one chromatid as in figs. 4 c, 4 d, 5, 6, and 7 make it clear that to be of significance as indicating the formation of a permanently branched chromosome, observations of any given lateral translocation must be made in more than one cell generation. The chromatid lateral translocations will be terminal translocations in the second somatic cell generation, and will involve duplication and deficiency. Their survival or loss will presumably depend upon the position of the spindle attachments ; if the break and rejoin occurs at this point in both chromosomes, as it appears to have done in fig. 5, both of the new type chromatids as well as the original one may be able to survive. If there is a spindle attachment on only one of the united chromosomes, and if it is situated elsewhere than at the break, one of the new type chromatids will lack an attachment; if there are attachments on both the united chromosomes one new type chromatid will have two attachments and sooner or later will break during mitosis. The interpretation of fig. 4 d which is given in diagram $4 d_1$ shows that " reciprocal translocations " may arise as a result of lateral chromatid translocations which are intercalary in both the united chromosomes.

It may further be noted that chromatid translocations between homologous chromosomes (including but not necessarily limited to exactly reciprocal interchanges or somatic crossing-over) would give rise in succeeding nuclear generations to homozygous tissues in a heterozygous plant. This is a third possibility which may be added to the two mentioned by Horlacher and Killough (1933) as possible explanations of their discovery of homozygous bolls in irradiated heterozygous cotton plants. Chromatid translocations can also result in duplication in later nuclear generations. The appearance of dominant characteristics in parts of an X-rayed homozygous recessive plant may therefore not *prove* " progressive " gene mutation, since in some cases (reviewed by Sewall Wright, 1934) duplication of recessive genes can produce the dominant phenotype. Clearly, tests for the location of genes in subsequent generations of X-rayed organisms, such as have been made by Muller and others on *Drosophila*, are necessary before any very definite conclusions can be drawn regarding the nature of induced changes.

Painter and Muller (1929) found that in all translocations analysed in their X-rayed *Drosophila* it was the broken end of the fragment that was attached. This would, of course, follow if terminal translocations are originally lateral chromatid translocations. There is no obvious reason why irradiation should cause ends of chromosomes to enter into per-

manent union; they apparently never do so following meiotic association. On the other hand, our observations indicate that whenever a break occurs in a single chromatid the two ends possess an unsatisfied attraction force and tend to unite with any chromatid ends which happen to be near them. There is no obvious reason why they should not also often reunite. The attachment of an unbroken end, noted as a rare occurrence in *Drosophila* (*cf.* Muller, 1932) and direct inversion of terminal segments might, from our observations, be expected to occur only when a fragment had been broken off from *one* of the chromatids. " Insertion " would follow a single break in a chromatid of one chromosome and two breaks in a non-homologous chromosome that chanced to be lying near it.

The observation of chromatid translocations indicates that if chromonemata were broken at a chiasma during the heterotypic division they possibly could rejoin, as postulated by Sax (1930) (though probably only if the break should occur before the matrix is accumulated). There is, however, much cytological evidence against Sax's hypothesis as a general explanation of normal crossing-over (*cf.* Hearne and Huskins, 1935, and Huskins and Smith, 1935). The mechanism he postulates may well be a cause of unequal crossing-over.

Darlington (1932) maintains that chromosomes are longitudinally single bodies in the somatic anaphase or telophase, and that they split during the resting stage in readiness for the succeeding anaphase separation. Similar views were held by Belar (1929) and Belling (1935). On this basis Darlington has built up a most attractive theory relating meiosis to mitosis. Were it valid many complications of chromosome behaviour would be simplified. Kaufmann (1926), Sharp (1929), and many others have, however, found that large plant chromosomes are longitudinally double in somatic anaphases and Stern (1934) has genetic evidence of four-strand somatic crossing-over in *Drosophila melanogaster*. Darlington (1926) showed quadripartite structure in pollen-grain metaphase chromosomes of the Scilleœ, but later discounted these observations. We cannot accept his interpretation of root tip or pollen grain somatic anaphase chromosomes as single bodies, but it seems possible that the striking disagreement between other leading cytologists may not be as serious as it at first sight appears. Belling emphasized that his observations were mainly upon cells " which would not normally divide again " and some of Belar's observations were on similar material. On the " mitosis-meiosis " hypothesis (Huskins, 1933), which is a modification of Darlington's (1932) most stimulating " precocity theory," it is an interesting coincidence that in salivary gland cells of *Drosophila* which

have undergone their last division Painter (1933) found the chromosomes to be paired as closely as in meiosis. Before generalizations are made on the doubleness or singleness of anaphase chromosomes it may be well to consider the possibility of differences existing in different tissues or different organisms. There are clearly differences in this regard in certain tumour cells. It would not be very surprising if visible splitting of the chromosomes, which must be in part a growth process, were restricted or inhibited in last division cells which are at the end of an active phase of multiplication—McClung (1927) especially has noted the cytoplasmic poverty of last spermatogonial cells in the Orthoptera. If this were found to occur, many present discrepancies in observations would be resolved. This problem is discussed in further detail by Huskins and Smith (1935). Both in pollen-grain somatic (*cf.* fig. 3) and meiotic (*cf.* Huskins and Smith, 1935) chromosomes of *Trillium*, the " attachment region " as well as the rest of the chromosomes is clearly quadripartite at metaphase and double at anaphase. Sharp (1929) has shown anaphase doubleness at the attachment region in root-tip chromosomes of *Trillium*. Darlington (1932), however, assumes singleness prior to metaphase, and division of the spindle attachment at metaphase in mitosis and later in meiosis.

Mather and Stone consider that their irradiation experiment proves Darlington's contention that chromosome splitting occurs in the " resting stage," since only " chromosome " breaks were found. Our results, as shown, are in striking contrast, and it appears that it may be differences in the method of approach and the staining technique which are responsible for the differences in observation. The occurrence of chromatid breaks in our material, though completely in line with our observation that the splitting occurs in the prophase of the division preceding separation, does not, however, prove it, since the irradiation treatments from which the results could be analysed in detail were made only at telophase or the early resting stage. Our results, both observational and experimental, are, however, very strongly against Darlington's view, and closer analysis of Mather and Stone's observations shows that their interpretations are not necessarily valid.

The two chromonemata lie in close juxtaposition through the division cycle prior to their separation, and they must at many points both be in the plane of irradiation. Whether X-rays break chromosomes through direct physical action or by causing molecular disruption, they might therefore be expected to produce both chromosome and chromatid breaks. It seems obvious that chromatid breaks are strong evidence, though not proof, of doubleness at the time of irradiation, whereas

chromosome breaks are not evidence of singleness. This is particularly true if chromatid breaks may sometimes rejoin.

Mather and Stone (1933) have summarized the evidence for and against the possibility that attachment constrictions may arise *de novo* in fragmented chromosomes as claimed earlier by Darlington (1929) and others. Their evidence is against *de novo* origin. McClintock (1932) observed one case in which breakage occurred within the attachment region; both fragments then possessed functional attachments. Our fig. 5 indicates a similar occurrence; otherwise, like Mather and Stone, we find no new attachments produced. The occurrence of a chromatid break within the attachment region provides further evidence against Darlington's (1932) assumption that in mitosis the spindle attachment does not divide until the metaphase.

SUMMARY

Microspores of *Trillium erectum* L. were irradiated in the homœotypic telophase or early " resting stage," of the pollen tetrads. The six anthers in a bud are usually almost synchronous in their division stages. One anther was removed immediately after irradiation and the remainder at 2–3 day intervals. It was thus possible to determine with fair accuracy the stage of division at the time of irradiation, and to be certain that the observations were made in the first division cycle after irradiation. The chromosomes were stained to show their internal, chromatid structure.

No morphological effects were observed immediately following irradiation but mitosis was delayed.

X-radiation causes breaks and translocations of either whole chromosomes or of their constituent chromatids. This applies equally to the region of the " attachment constriction."

Lateral translocations of chromosome fragments on to a broken chromatid were found with high frequency in the first mitotic prophase following irradiation. In the succeeding nuclear generation these will, of course, appear to be terminal translocations.

" Constrictions," apparently similar to those reported by other observers as arising after irradiation, were found upon de-staining to be merely chromatid breaks.

Breaks occurring in chromatids will not be obvious *before* anaphase in preparations so stained that the chromonemata are not differentiated from the surrounding matrix and they may not be seen until the next division. This explains, in part, the generally observed " delayed action " of X-rays in causing fragmentation.

The somatic chromosomes of *Trillium* microsporocytes, including the " attachment constriction," are longitudinally double at all stages except just prior to anaphase separation when they are 4-partite.

" Trabants " have been found to arise *de novo*.

One example of ring formation was observed.

The observations are discussed in relation to certain genetic evidence on the nature of chromosome rearrangements and mutations.

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Studies on the Hypophysectomized Ferret
IX—The Effect of Hypophysectomy on Pregnancy and Lactation

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[PLATES 5, 6]

I—INTRODUCTION

Animals of several different species have been hypophysectomized during pregnancy. Aschner (1912) found that hypophysectomy in the pregnant dog led to abortion. Pencharz and Long (1931, 1933) in their study of the rat, reported that the fœtuses fail to become implanted if the operation is performed on the fourth day of pregnancy; operation from the seventh to tenth days of pregnancy results in resorption of the fœtuses, while from the eleventh to twentieth day it is followed by death of the mother at the end of a prolonged gestation or by the birth of dead or living young. Selye, Collip, and Thomson (1933, *a*, *b*) have extended these studies both on the rat and mouse. They found that hypophysectomy during the second half of pregnancy in the rat does not usually prevent the birth of the young, although gestation is prolonged, nor does it prevent the development of the mammary gland. Milk secretion begins at parturition but ceases within 26 hours. In the mouse, operation during the second half of pregnancy does not lead to abortion, although it was uncertain whether or not the gestation was prolonged because the pregnancies were undated. The mice resembled the rats in that temporary milk secretion occurred. These authors also report (1934) that a guinea-pig hypophysectomized on about the fourteenth day of pregnancy carried a living fœtus when killed 8 days later. Pencharz and Lyons (1934) have made a more thorough study of the guinea-pig and find that hypophysectomy at 5 weeks pregnant results in resorption, but at 6 weeks does not interfere with the course of gestation. Also, as in the rat, a few hours milk secretion follows parturition. Allan and Wiles (1932) found that hypophysectomy in the cat late in pregnancy

does not cause interference with the parturition mechanism. Nine animals all delivered living or dead fœtuses. Two litters, however, were definitely premature. White (1933–34) reported abortion following hypophysectomy in the rabbit. The writer (1935) also found that in the cat abortion may result from hypophysectomy. Milk secretion in the cat may occur for longer than in the rat or mouse; in one animal milk could be expressed from the glands as late as 6 days after operation.

The present paper deals with the effects of hypophysectomy in the pregnant ferret.

II—METHODS AND MATERIALS

Animals—All the animals were purchased from dealers, with the exception of six distemper-immune ferrets from the Institute Farm Laboratories, which were kindly given to me by Dr. P. P. Laidlaw, F.R.S. They were kept until in full œstrus and then mated, so that all pregnancies were exactly dated.

Operative Technique—The operation was performed according to the procedure described by Hill and Parkes (1932). At autopsy the sella turcica of each animal was carefully examined for remnants of anterior lobe. In no instance were macroscopic portions found. This observation was checked by serial section of the pituitary fossæ of all animals except two. In control operations, the usual procedure was followed exactly, except that the pituitary was not removed.

Material Available—Excluding a few ferrets which died during the course of the experiment, results have been obtained on 20 animals. Of these 4 were hypophysectomized at the 21st day of pregnancy and 9 at the 35th, 3 of which were injected. Six were used as controls, 2 being ovariectomized and the other 4 subjected to control exposure of the pituitary. The remaining animal was hypophysectomized while lactating. Details of these experiments are given in Table I. It was considered necessary to perform the control operations, since it was possible that the mere shock of the manipulation might bring about abortion.

Histology—The whole mounts of mammary glands were prepared in the usual manner. In most cases half of one gland was embedded, cut at 10 μ and stained with hæmatoxylin and eosin.

Extract—An extract containing the gonadotropic and mammary gland-stimulating principles of the anterior hypophysis was kindly

prepared for me by Mr. I. W. Rowlands. It consisted of the alcoholic precipitate of an alkali extract of acetone-desiccated pituitary anterior lobe, designated T20B. In order to insure potency of the extract only a small quantity of the dry powder was put into solution at a time.

III—CONTROL OPERATIONS

Control Operations—Two of the animals used as operation controls, HFP28 and HFP31, had normal litters on the 41st day of pregnancy, the other two, HFP27 and HFP29, had them on the 42nd day. These periods of gestation are strictly normal for the ferret (Hammond and Marshall, 1930). HFP27 and HFP28 each lost one of their young, but otherwise all were suckled successfully. Thus it can be stated with certainty that the manipulation involved by hypophysectomy does not in itself lead to abortion, or to debility of the young.

Castrated Animals—The extensive literature dealing with the ovariectomy of pregnant animals will not be dealt with here; in most species removal of the ovaries during pregnancy results in abortion or resorption of the fœtuses. Two ferrets were ovariectomized at the 35th day of pregnancy and studied in conjunction with the hypophysectomized animals. HFP20 aborted eight young 3 days after operation, but failed to rear them although the mammary gland secreted heavily. It is probable that the young are not viable at 38 days. The other animal, HFP23, had a premature litter of eight at 40 days post-coitum, and successfully reared four of them.

IV—EFFECT OF HYPOPHYSECTOMY ON THE COURSE OF PREGNANCY

Hypophysectomy at the 21st day of Pregnancy—Of the four ferrets hypophysectomized at this stage, HFP26 aborted two partially reabsorbed fœtuses 12 days later, while HFP21 and HFP30 died 3 and 4 days respectively after operation when resorption had already started. The remaining animal, HFP32, killed 9 days after removal of the pituitary, contained one living fœtus.

This series is obviously inconclusive, but the experiments make it probable that hypophysectomy of the ferret on the 21st day of pregnancy causes termination of gestation.

Hypophysectomy at the 35th day of Pregnancy—Six ferrets hypophysectomized at 35 days pregnant were not further treated; delivery occurred 3–8 days after operation. Four of these had definitely premature

litters and only two went to term. In HFP7 parturition occurred on the 42nd day of gestation and in HFP11 on the 42nd and 43rd day.

As will be seen from Table I none of these young lived for more than one day. With premature young this is not surprising, but the death of those born at 40 and 42 days post-coitum requires explanation. This probably lies in the fact that lactation failed to occur in the hypophysectomized animals. In spite of the removal of the pituitary there was no impairment of maternal care in these animals. The retrieving instinct, for instance, was very obvious.

Selye, Collip, and Thomson (1933, *a*) have reported that hypophysectomy prolongs the morphological life of the corpora lutea. This phenomenon was not found in the ferret. The data given by Hammond and Marshall (1930) show that the corpus luteum of the ferret decreases very rapidly in size in the last week of pregnancy and afterwards. My own observations are in keeping with their results. From 4 to 5 weeks post-coitum the corpus luteum is at its maximum, measuring about 2 mm in diameter. Shortly after parturition it has decreased to a little over 1 mm, and had lost all appearance of activity. At 2–3 weeks post-partum it is reduced to fibrous remains, which may be little over 0·5 mm in diameter. The hypophysectomies described above were therefore carried out at the time of maximum development of the corpus luteum, and any prolongation of their histological integrity would have been readily observed. Microscopical examination of the ovaries of HFP9, HFP7, and HFP11, 10, 15, and 20 days after delivery of the young, showed that the course of atrophy of the corpora lutea had been entirely normal and that in this particular the ferret differs from the rat.

Hypophysectomy at the 35th day of Pregnancy with Administration of Anterior Lobe Extract—Three animals, HFP16, HFP17, and HFP19, were given 50 mg T20B daily for three successive days, the injections being started on the day following the operation. Administration of the extract was not continued further as it was thought that it might unduly prolong pregnancy. HFP17 aborted on the day following the operation, *i.e.*, the day of the first injection, so that this animal could hardly have been affected by the extract. HFP19 delivered on the 42nd day of pregnancy and HFP16 on the 44th; the latter thus definitely went beyond term. The longest periods of gestation of the six non-injected animals operated on at the 35th day of pregnancy were two of 42 days, and it is very likely from these results that the injection of the pituitary extract had some effect in maintaining the pregnancy.

TABLE I

No. of ferret	Days pregnant when operated	Operation	Amount anterior pituitary left	Delivery, days after operation	No. of young	Treatment	Course of experiment	Time mammary gland obtained, days after delivery
HFP21	21	Hypophysectomy	None	—	—	—	Animal died 3 days after operation. Resorption occurring.	—
HFP26	21	"	"	12	2 (abnormal)	—	Abortion...............	15
HFP30	2I	"	? fragment	—	1 embryo	—	Animal died 4 days after operation. Resorption occurring	—
HFP32	21	"	Not sectioned	—	1 embryo	—	Killed 9 days after operation, 1 living fœtus	—
HFP7	35	"	None	7	1	—	Young dead 1 day later	15
HFP8	35	"	"	4	9	—	7 young alive and 2 dead; all dead 1 day later	5
HFP9	35	"	"	5	13	—	6 young alive and 7 dead; all dead 2 days later	10
HFP15	35	"	"	4	8	—	4 young alive and 4 dead; all dead 1 day later	1

HFP10	35	"	? fragment	3–4	7	—	5 young alive and 2 dead; all dead 1 day later	9–10
HFP11	35	"	None	7–8	7	—	6 young alive and 1 dead; all dead 1 day later	19–20
HFP16	35	"	"	9	4	Anterior lobe extract	4 young alive; all dead 1 day later	14
HFP17	35	"	Not sectioned	1	2	"	2 young alive; 1 died after 1 day and other after 5 days	11
HFP19	35	"	None	7	8	"	8 young alive; 1 died after 1 day and others after 2 days	5
HFP27	35	Control "hypophysectomy"	All	42	7	—	6 alive; 1 dead; suckling ..	—
HFP28	35	"	"	41	6	—	5 alive; 1 dead; suckling ..	—
HFP29	37	"	"	42	8	—	8 alive; suckling	—
HFP31	37	"	"	41	7	—	7 alive; suckling	—
HFP20	35	Ovariectomy	"	3	8	—	4 young alive and 4 dead; all dead 1 day later	10
HFP23	35	"	"	5	8	—	8 young alive; 4 died 2 days later	—
HFP13	Lactating 20 days	Hypophysectomy	None	—	9	—	Milk secretion ceased 2 days after operation and animal died 4 days later	—

V—THE EFFECT OF HYPOPHYSECTOMY ON THE MAMMARY GLAND

Selye, Collip and Thomson (1934) have performed a number of interesting experiments on lactation in the rat and mouse and their paper includes a good review of the literature on this subject. The behaviour of the mammary glands in the ferrets recorded in Table 1 is described below.

Hypophysectomy at the 21st day of Pregnancy—The mammary gland was obtained from HFP26, which was killed 15 days after abortion. The macroscopic preparation shows an extremely small and undeveloped gland, fig. 2, Plate 5. This gland, obtained at 36 days post-coitum and 3 days after abortion, is insignificant compared with a normal 35-day post-coitum gland, fig. 1, Plate 5, and shows quite definitely that hypophysectomy stops the normal development which takes place even during pseudo-pregnancy.

Hypophysectomy at 35th day of Pregnancy—In the normal animal abortion at a late stage of pregnancy results in the appearance of milk in the gland. In the animals which had premature or even full-term litters after hypophysectomy there appeared to be no milk in the glands, as determined by traction on the nipple, except for a slight temporary secretion in HFP8. In HFP15, killed 1 day after delivery of the young at 39 days post-coitum, the gland had not the appearance of a secreting gland, fig. 3, Plate 5, and on histological examination was seen to be quite inactive. Except for a few ducts, the glandular tissue was almost solid, the alveoli being very small and undistended, fig. 10, Plate 6. The subsequent history of the mammary gland after hypophysectomy late in pregnancy was studied in HFP8, HFP10, HFP9, HFP7, and HFP11, killed at 5, 10, 10, 15, and 20 days respectively after parturition. This series shows a gradual decrease in the expanse and thickness of the mammary glands, figs. 4 and 5, Plate 5.

Histologically, the first three of the glands showed traces of secretion in the alveoli, fig. 11, Plate 6, but as there was no thickening of the glands, this had the appearance of being due to atrophic breakdown of the alveoli.

Involution of the mammary gland takes place fairly rapidly even in a normal animal after the cessation of the suckling stimulus, but the gland obtained from a control (HFP12, not recorded in the table), 10 days after delivery and 3 days after the end of suckling, is large in comparison with those recorded above, and contains large alveoli and ducts distended with milk. Similarly the gland from HFP20, a castrate, 10 days after

abortion and 9 days after suckling resembles the normal HFP12, fig. 6, Plate 5. This gland was entirely different from that of HFP10, *i.e.*, 13 days after hypophysectomy and 10 days after delivery. The regression of the mammary gland in the pregnant ferret after hypophysectomy is thus superficially more rapid than after removal of the suckling stimulus. This is probably due to the fact that after hypophysectomy the gland fails to undergo the thickening and secretory activity normally found at parturition, and that therefore the problem of regression is much more simple.

Hypophysectomy on the 35th day of Pregnancy with Injection of Anterior Lobe Extract—HFP16, HFP17, and HFP19, the three animals hypophysectomized at 35 days pregnant which received 150 mg each of T20B in 3 days, showed at the end of this time really remarkable development of the mammary glands. The glands were gorged with milk and showed as two distended ridges on the animals' belly. If the animals struggled while being held milk shot out from their teats, a condition rarely if ever found in the normal lactating animal. As each animal was differently treated a brief summary of their histories is given below.

*HFP*17.
    June 21—Hypophysectomy.
    June 22—50 mg T20B given. Aborted two fœtuses.
    June 23—50 mg T20B given. Young still alive.
    June 24—50 mg T20B given. One dead. Mammary gland well up and secreting.
    June 27—Other young dead.
    July 3—Killed. Mammary gland still large.

*HFP*16.
    June 21—Hypophysectomy.
    June 22—50 mg T20B given.
    June 23—50 mg T20B given.
    June 24—50 mg T20B given. Mammary gland well up and secreting.
    June 28—Gland going down rapidly.
    June 30—Delivery of four young. Given 50 mg T20B.
    July 1—Given 50 mg T20B.
    July 2—In evening all young dead. Mammary gland without milk.
    July 3—Milk readily expressible from gland.
    July 9—Gland atrophied. No milk.
    July 10–13—50 mg T20B daily. On 13th milk readily expressed from gland.
    July 14—Animal killed. Mammary gland large.

*HFP*19.

> June 21—Hypophysectomy.
> June 22—50 mg T20B.
> June 23—50 mg T20B.
> June 24—50 mg T20B.   Mammary gland large and secreting.
> June 27—Gland down and hard.
> June 28—Eight young born.   50 mg T20B given.
> June 29—Six young dead.   50 mg T20B given.   No milk from ducts
> > as yet.
> June 30—Other young dead.   Mammary gland enormous (see fig. 8,
> > Plate 5).

The results from these animals are interesting.   As far as we are aware milk secretion has not yet been produced in hypophysectomized animals. Selye, Collip, and Thomson (1933, *a*) found that " removal of the luteinized ovaries will lead to milk secretion in the fully developed gland of the A.P.L. treated rat, but only in the presence of the pituitary."   These same authors (1934) state that they were unable to maintain lactation in hypophysectomized rats by means of pituitary implants.   They do not state how much hypophyseal tissue was given, but it may be impossible to give sufficient pituitary material by this method to obtain the necessary stimulus to maintain lactation.   Certainly, by means of our somewhat crude extract abundant lactation was readily brought about in the only three animals injected.   It was hoped that hypophysectomized injected mothers would rear their young as a result of treatment but this was not realized.   HFP17 aborted on the 36th day of pregnancy when the young were certainly not viable and the mammary activity was of no value. This animal was killed 9 days after the last injection of anterior lobe extract.   The mammary gland was still large and was certainly not undergoing regression from its previous condition of activity any faster than does the gland of the normal lactating ferret after the premature removal of the young, figs. 6 and 7, Plate 5.

Administration of anterior lobe extract to HFP16 and HFP19 had to be stopped after three injections because the treatment seemed to be prolonging gestation.   HFP19 was injected again immediately after parturition, but milk could not be expressed from the gland until 2 days later, by which time the young had died.   Fig. 8, Plate 5, shows the enormous development of the gland compared with that of the untreated hypophysectomized animal.   Similarly, HFP16 was injected soon after delivery, but here again milk secretion was initiated too late to save the young.   This animal, however, was utilized further ; the mam-

mary gland was allowed to atrophy for the second time after being brought to the milk-secreting stage by injection and then again treated for 4 days. Its growth did not appear to be as extensive as on the previous occasions, but milk could readily be expressed from the teats after 4 days treatment.

From these experiments it would appear likely that the injection of anterior lobe extract at an opportune moment should bring about milk production in a hypophysectomized animal in time to save the young.

Histologically the glands from these animals are significant. Those of HFP19 and HFP16, killed immediately after the end of a series of injections, showed tremendous activity. The alveoli and ducts were large and distended with milk, fig. 12, Plate 6. HFP17 was killed 11 days after abortion and 9 days after the last injection. Although the gland was fairly large, the alveoli were undergoing involution. The ducts were still large but contained little milk.

Hypophysectomy during Lactation—Established lactation is regularly and rapidly terminated by hypophysectomy in both rats and mice (Selye, Collip, and Thomson, 1934). This undoubtedly holds true for the ferret. HFP13 with nine young was hypophysectomized 20 days after delivery. Lactation stopped 2 days after the operation.

VI—SUMMARY

Hypophysectomy at the 21st day of pregnancy in the ferret may result in abortion or resorption of the fœtuses. Hypophysectomy at the 35th day of pregnancy is followed by the delivery of dead or living young from 3–8 days later. Lactation is rarely initiated and never continued. The young invariably die.

The injection of an extract of anterior hypophysis caused hypertrophy and secretion of milk in the mammary glands of three hypophysectomized animals, both before and after delivery of the young.

Exposure but not removal of the pituitary at the 35th or 37th day of pregnancy caused no interference with the normal mechanism of parturition and lactation.

Removal of the ovaries of two pregnant animals (35th day) resulted in premature parturition. Young born at 40 days post-coitum were reared, but not those born at 38 days post-coitum.

Hypophysectomy of a ferret during lactation caused termination of milk secretion.

DESCRIPTION OF PLATES

PLATE 5

Macro-photographs of whole mounts of mammary glands.   × 1

FIG. 1—Mammary gland of COF47, 35th day of normal pregnancy.

FIG. 2—Mammary gland of HFP26, 36 days post-coitum, 3 days after abortion following hypophysectomy at 21 days. Mammary development has not proceeded after hypophysectomy.

FIG. 3—Mammary gland of HFP15, one day after premature birth and 5 days after hypophysectomy at 35 days pregnant. Lack of thickening normally found after parturition.

FIG. 4—Mammary gland of HFP10, 10 days after premature birth and 13 days after hypophysectomy at 35 days pregnant. Gland in regression and showing lack of continuity normally found in secreting gland.

FIG. 5—Mammary gland of HFP11, 20 days after parturition and 27 days after hypophysectomy at 35 days pregnant. Gland much regressed.

FIG. 6—Mammary gland of HFP20, 10 days after premature birth and 13 days after ovariectomy at 35 days pregnant. Nine days after death of young. Gland still well up.

FIG. 7—Mammary gland of HFP17, 11 days after premature birth and 12 days after hypophysectomy. Nine days after last of three injections of anterior lobe extract; gland still well up.

FIG. 8—Mammary gland of HFP19, 2 days after parturition and 9 days after hypophysectomy at 35 days pregnant. One day after last anterior lobe extract injection. Gland secreting very heavily.

PLATE 6   × 30

FIG. 9—Mammary gland of COF47, *cf.* fig. 1.

FIG. 10—Mammary gland of HFP15, *cf.* fig. 3, showing quiescence.

FIG. 11—Mammary gland of HFP9, showing traces of secretion.

FIG. 12—Mammary gland of HFP19, *cf.* fig. 8, showing great secretory activity.

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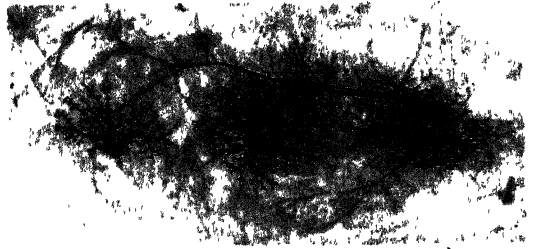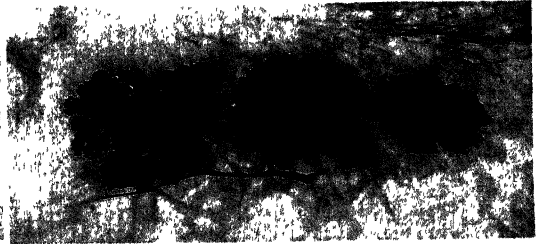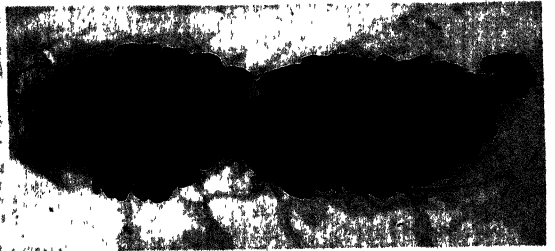
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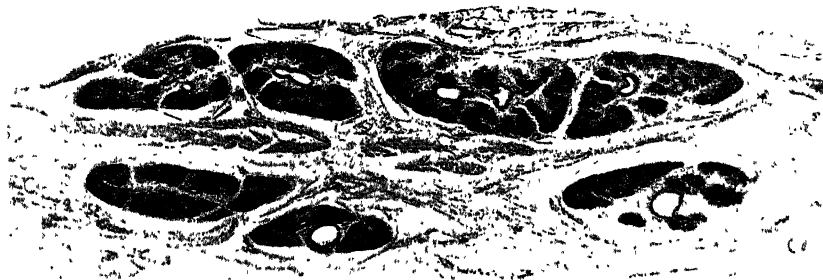
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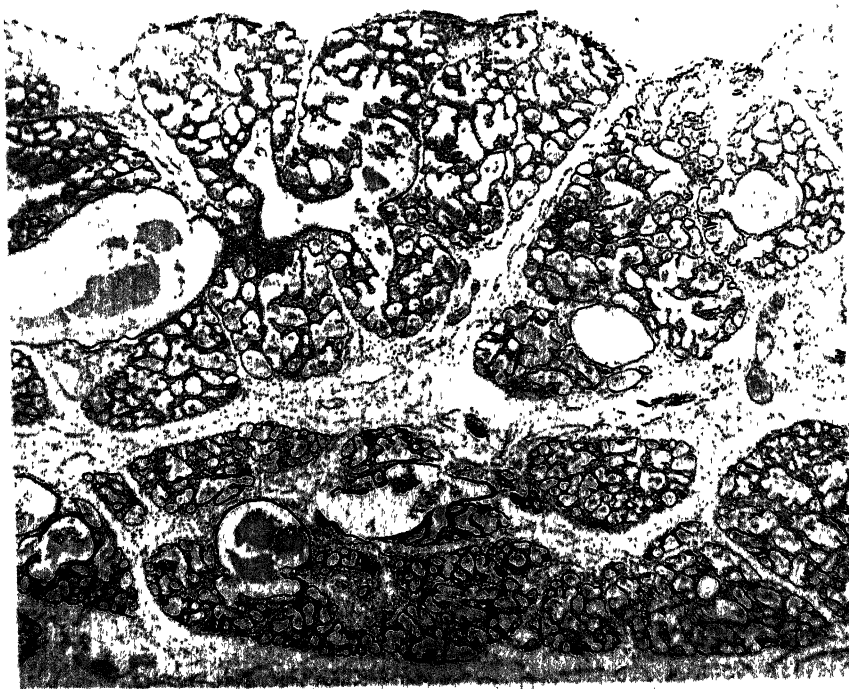
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Hypophysectomy of the Cat

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[Plates 7-11]

I Introduction

The effects of hypophysectomy in the cat have not yet been described in any detail. McLean (1928) removed the pituitary of the cat by a buccal approach, but did not deal with the specific changes in the reproductive organs. Allan and Wiles (1932) carried out retropharyngeal and buccal hypophysectomies in pregnant cats with a view to determining the rôle of the posterior lobe in parturition. They give no details as to the effect of the operation on lactation or on the subsequent history of the reproductive organs.

In view of the importance of obtaining information about the effect of hypophysectomy in as many species as possible, it seemed desirable to extend the work of these authors on the female and also to investigate the male cat. Hypophysectomy was performed by a parapharyngeal approach similar to that used by Smith (1930) in the rat, and M. Hill and Parkes (1932) in the ferret. This operation differs somewhat from that used by Allan and Wiles, and has, moreover, been facilitated by the use of a tracheal cannula. For comparison with the changes in the reproductive organs of the hypophysectomized cat, information as to the normal reproductive processes of this animal has been obtained by an examination, in conjunction with Dr. Parkes, of a large series of normal material, which will form the basis of a separate study.

II Technique and Material

The animals used in this work were purchased from dealers and consequently their history is only known from the time of coming into the laboratory. They were all obviously adult, with the possible exception of CH 17 and CH 29, and in good health. Since the resistance of hypophysectomized animals is much below normal all operated cats were kept in a special warm room with a temperature of 75–80 °F.

*Operative Procedure**—The animal, under full anæthesia with chloroform and ether, is stretched out in the dorsal position, head towards the operator, and a tracheal cannula inserted. An incision about 5 cm long is made in the neck, running backwards from 1 cm behind the point of the jaw and diverging a little to the right of the mid-line. The mylohyoid and digastric muscles are separated at their line of junction and retracted. In the angle formed by the nerve and the external maxillary artery the hyoid bone can be felt under the thin styloglossus muscle. The operation is facilitated by the removal of the epihyal, which apparently results in no ill effects. The surface of the bulla immediately beneath it is reached by breaking through the connective tissue and periosteum. A small vein issuing at the corner is sometimes ruptured but the bleeding is readily checked by the application of a piece of crushed muscle. The surface of the sphenoid is then reached near the styliform process of the bulla. The bone is freed of muscle by means of swab dissection which is continued anteriorly until the pterygoid canal is seen or felt. The bone is now ready for drilling, fig. 2. A small vein, as mentioned by Allan and Wiles, issues near the place where the hole should be made, and this can be used as a landmark. The pharynx and connective tissue from the surface of the bulla and sphenoid are held aside by a stout curved retractor, fig. 2. The removal of the hyoid bone makes it possible to carry out the whole of this manipulation from immediately above the site of the drill hole and a much better view can be obtained of the pituitary than when it is necessary to tunnel anteriorly as in Allan and Wiles' operation.

The pituitary body in the cat, partially covered by a prominent dorsum sellæ, fig. 4, Plate 7, lies in a deep fossa which is bounded laterally by the two cavernous sinuses lying in connective tissue. The posterior lobe is relatively smaller than in the hedgehog or guinea-pig and is not embedded deeply enough in the anterior lobe to divide the latter into two parts.

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* Since the operation is almost identical with that on the ferret, and is somewhat similar to that of Allan and Wiles, an outline description only is given.

AREA DRILLED for HYPOPHYSECTOMY

STYLIFORM PROCESS of BULLA

TYMPANIC BULLA

OCCIPITAL

BASI-SPHENOID

PRE-SPHENOID

MANDIBLE

FORAMEN

ZYGOMATIC ARCH

PALATINE

B C Rillyson
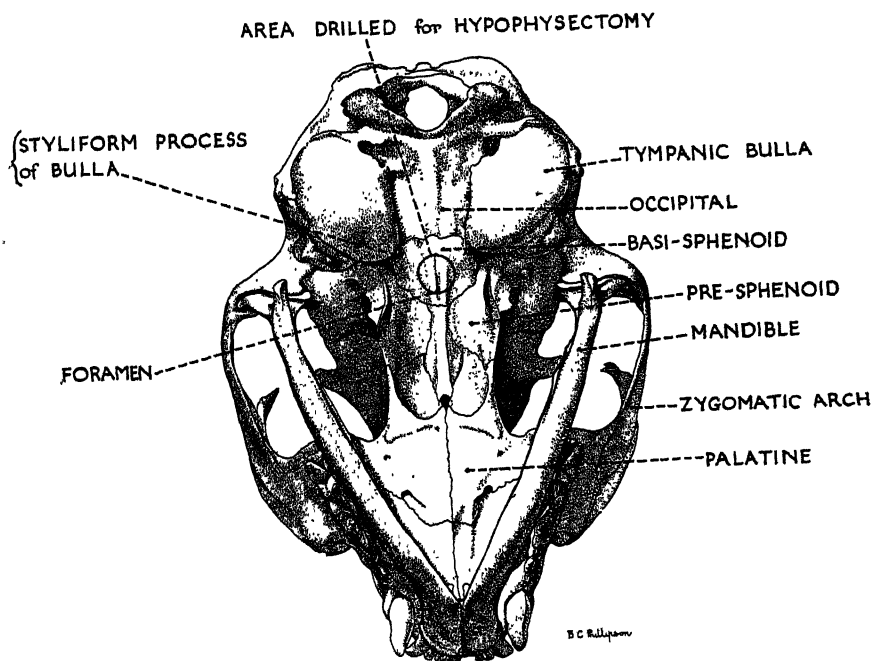
FIG 1—Showing ventral surface of cat skull and area for drilling

HYPOGLOSSAL NERVE

LINGUAL ARTERY

EDGE of TYMPANIC BULLA

EXTERNAL MAXILLARY ARTERY

MYLO-HYOID

STYLIFORM PROCESS

PTERYGOID CANAL

DIGASTRIC

B C Rillyson
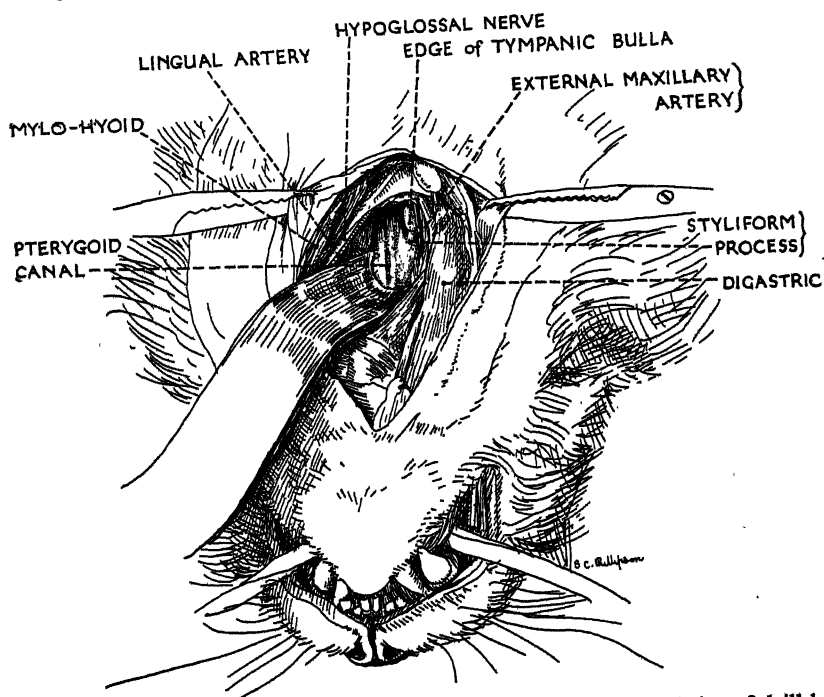
FIG. 2—Showing operative approach with retractor in place and site of drill hole

The pars intermedia is unusually large. From the operative approach the gland visible is mainly anterior lobe with a small portion of the pars nervosa visible as a slight crescent at the posterior margin of the drill hole. The skull is spongy for a carnivore and blood tends to ooze out when the drill hole is made. For adult cats a number 16 dental burr is used. The exposure and removal of the gland is as described by Allan and Wiles.

After removal of the gland the cavity is closed in the ordinary way. Anæsthesia is then lightened until respiration is very strong, the tracheal cannula removed, and the open ends of the trachea swabbed clean and brought firmly together by means of silk. It is important to have the animal breathing strongly at this stage, otherwise difficulty may be experienced in clearing the air passage of mucus.

Material Available—Twenty-five cats were successfully hyophysectomized and recovered from the operation. At autopsy, several of these were seen to have fragments of the anterior lobe still *in situ* and were discarded, except for one which is considered in the pregnancy series. Histological examination of the sellæ turcicæ showed traces of what might have been anterior lobe tissue in some of the remaining cats, but examination of the organs showed that in only two (CH 34 and CH 38) could the remains have been physiologically active. These two animals were discarded. The remaining female cats, 9 non-pregnant, 4 pregnant and 3 lactating at the time of operation, were either hypophysectomized without doubt or possessed only insignificant traces of anterior lobe to which no physiological function could be attributed. In addition, three completely hypophysectomized males were obtained at various stages after operation.

Chemical Methods—The blood for the glucose determinations, which were made by the Hagedorn-Jensen method, was collected from the marginal ear veins at hourly intervals, $0 \cdot 1$ cc of blood being used for each determination.

Histological Technique—All organs, with the exception of the kidneys and spleen which were weighed fresh, were fixed and then weighed at the 70% alcohol stage of the upgrading process. Bouin's fluid was used as a fixative and hæmatoxylin and eosin for staining all tissues except the adrenals. One adrenal was treated in the usual manner, the other was fixed in Ciacio's fluid and stained for lipoids with Scharlach R. Sections were cut generally at 5, 7 or 10 μ. The pituitary fossæ of all operated animals recorded below were sectioned serially in order to determine if

removal of the anterior lobe had been complete. The mammary glands were prepared in the usual way with the exception of those of CH 46 which were photographed unstained.

III General Effects of Hypophysectomy

Immediate Post-operative Effects—The animals generally recovered well from the operation, the females giving less trouble than the males. Whether this is due to a real sex difference or not, is difficult to say. As a precaution the males were generally injected with an active anterior pituitary extract for the critical three or four days immediately following the operation. Glucose and physiological saline, too, were occasionally given at the time of operation.

Hypophysectomized animals of long standing eat and drink well, but appear apathetic and less playful than normals.

Body Weight—Records were kept of the weights of the animals at the time of operation and at autopsy. Usually the animals lost some weight; 7 of 9 non-pregnant females and 2 of 3 males showed a decrease in weight, and the others a slight increase, when killed at autopsy. It is probable that a sharp decrease in weight occurs immediately after operation, and then a gradual return to a slightly subnormal level.

Temperature—From a limited number of observations it would appear that the hypophysectomized animal has a slightly lower body temperature than the normal; the average of our observations being 39·4° for the hypophysectomized cat and 39·8° for the normal. A lower temperature in hypophysectomized rabbits has been reported by White (1933).

Loss of Hair—As in rats, ferrets, and rabbits, removal of the hypophysis apparently results in a loss of hair. No definite shedding of the coat occurs, but the animals lose hair more readily than is normal.

Resistance to Insulin—Opportunity was not found to make any detailed study of the carbohydrate metabolism after hypophysectomy, but certain observations have been made on the sensitivity of operated animals to insulin. That hypophysectomized animals are hypersensitive to insulin is well known. Geiling, Campbell and Ishikawa (1927) and Houssay and Biasotti (1931) have shown this for the dog, Corkill, Marks and White (1933) for the rabbit, and Hartman, Firor and Geiling (1930) for the monkey. The number of our observations is small, but they clearly indicate that the hypophysectomized cat, too, is hypersensitive

TABLE 1—EFFECT OF INSULIN IN THE NORMAL AND HYPOPHYSECTOMIZED CAT

No. of cat	Body weight, kg	Days after hypophy-sectomy	Amount of insulin given, units	Blood glucose, mg %		Effect
				Original	Lowest	
CH 30	2·1	52	1	106	38	Convulsions after 3 hours 30 minutes. Found dead in morning, i.e., 20 hours after start of experiment.
CH 39	2·9	32	2	—	—	Slight drowsiness and fall in temperature after 4 hours. No convulsions. Recovery good.
CH 28	2·2	65	3	88	31	Convulsions 1 hour 46 minutes. Death at 3 hours 25 minutes.
Cat 53	2·3	Normal	3	—	—	No obvious effects.
Cat 61	2·7	„	5	95	17	Co-ordination poor at 2 hours 15 minutes. Convulsions after 3 hours and marked fall in body temperature to 34°. Improved at 4 hours 3 minutes. Animal walking. After 7 hours temperature 38°.
Cat 58	2·5	„	10	—	—	Co-ordination gone at 1 hour 25 minutes. Convulsions 1 hour 45 minutes. Alive after 13 hours, but recovery not good.
Cat 62	2·8	„	10	—	—	Temperature dropped from 40° to 35·7° after 6 hours. Convulsions. 5 cc 20% glucose. Recovery complete in an hour.

to the action of insulin. Blood samples at hourly intervals were taken from cats after the administration of 1, 2, and 3 units of insulin respectively. Normal cats were given 3, 5, and 10 units of insulin, hourly blood samples being taken from the one receiving 5 units. The experiments are summarized in Table I, and two typical blood sugar curves are shown in fig. 3, where the operated animal received 1 unit of insulin and the normal one 5 units. The greater resistance of the unoperated animal is strikingly shown by the fact that its blood sugar dropped as low as 17 mg %
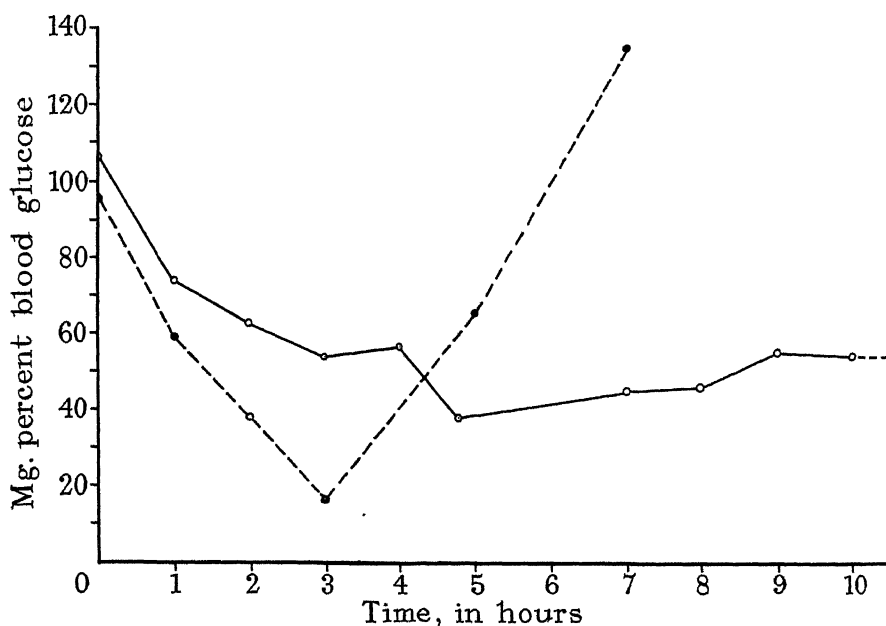
FIG. 3—Blood sugar glucose changes following administration of insulin to – – – – normal, and —— hypophysectomized cats

and its body temperature to 34°, and then recovered spontaneously to normal levels within a few hours.

Evidence for the fact that the sensitivity of operated animals to insulin increases with time is slight from our observations, but it may be mentioned that CH 30, 52 days after hypophysectomy, died after 1 unit of insulin, while CH 39, 32 days after operation, survived the administration of 2 units. The blood samples taken from the hypophysectomized animals before injection of insulin give no indication that a spontaneous hypoglycæmia occurs regularly in the hypophysectomized cat.

IV EFFECT OF HYPOPHYSECTOMY ON THE THYROIDS, ADRENALS,
KIDNEYS AND SPLEEN

The weights of the thyroids, adrenals, kidneys and spleen in 6 hypo-
physectomized female cats are shown in Table II, compared with the

TABLE II—EFFECT HYPOPHYSECTOMY ON THE WEIGHT OF VARIOUS
ORGANS OF THE CAT

No. of cat	Time of autopsy after hypophy-sectomy, days	Body weight at autopsy, kg	Weight of (gm)			
			Thyroids	Adrenals	Kidneys	Spleen
CH 46	22	2·5	0·175	0·276	19·1	3·75
CH 52	23	2·5	0·172	0·333	18·4	4·17
CH 39	32	2·9	0·164	0·304	18·4	4·31
CH 30	52	2·1	0·141	0·202	13·2	3·35
CH 28	65	2·1	0·117	0·249	16·5	3·60
CH 40	77	1·6	0·084	0·250	8·6	1·85
Average	—	2·3	0·142	0·259	15·7	3·50
Average per kg	—	—	0·062	0·113	6·8	1·52
6 normals—						
Average	—	3·0	0·214	0·506	22·5	6·34
Average per kg	—	—	0·071	0·169	7·2	2·17

averages for 6 normal female cats. The average weight of these organs
is appreciably lower in the hypophysectomized animals than in six
normals, but the average body weight of the normals was slightly higher
than that of the experimental ones at the time of operation, and dis-
tinctly higher than that of the experimental ones at autopsy. It is
debatable whether the weight at operation or that at autopsy is the best
basis for calculating the weight of the organs in relation to body weight,
but the latter may be considered as the safer. On this basis, there is a
slight and doubtfully significant drop in thyroid weight after hypo-
physectomy, a distinct drop of one-third in adrenal weight, an insignificant
change in the kidney and an appreciable fall in the weight of the spleen.
Considered on the basis of body weight at operation the changes would
be rather greater. These results are similar on the whole to those
reported by White (1933) on the rabbit.

Histology of the Thyroid—The thyroids of the hypophysectomized
cats were characterized by decrease in the size of the follicles, flattening

of the epithelium and relative increase in the amount of interfollicular tissue. The thyroid follicles of the normal cat are very variable in size, fig. 6, Plate 7, and there is some evidence that the atrophy following hypophysectomy decreases this variability. Thus the small follicles in CH 39, fig. 7, Plate 7, were remarkably constant in size.

Histology of the Adrenal—The size changes of the adrenal are reflected in the histological appearance of the cortex, which shows a loss of vascularity and definite cell shrinkage (*cf.* Smith, 1930; White, 1933). The latter is most marked in the outer region of the zona fasciculata which normally contains large cuboid vacuolated cells, figs. 8-11, Plate 8. In CH 46 and CH 28, 22 and 65 days after hypophysectomy, these cells are greatly reduced in size and sometimes can no longer be distinguished. The cortical atrophy, however, is generally insufficient to obliterate the differences between the three zones and preparations stained with Scharlach R suggest that, although there may be some increase in the stainable lipoids, the gland has not become completely inactive.

The adrenal medulla shows no perceptible alteration after hypophysectomy.

V Effect on the Female Reproductive Organs

Table III shows the material available for considering the changes in the female reproductive organs, the series extending from 14 to 77 days after hypophysectomy.

Ovaries—The ovaries in this series of animals were all much smaller than the ovaries of normal adults, but it is difficult to make an arithmetic comparison owing to variation in the size of the normal ovary during different stages of the cycle. They were all devoid of large normal follicles or active corpora lutea. Those of CH 14 and CH 26 contained cystic follicles, which would therefore appear to be maintained without the presence of the anterior pituitary. In all ovaries there were masses of young oöcytes, whose abundance was emphasized by the lack of large structures, and also large numbers of primordial follicles, consisting of a fully grown ovum surrounded by a few layers of solid follicular epithelium. Some ovaries contained in addition rather larger follicles in which the antrum had just appeared. The existence of these latter structures in cats which were undoubtedly hypophysectomized is of significance, since in the ferret the follicle is unable to acquire even the beginnings of an antrum in the absence of the hypophysis. The follicles in these cats

TABLE III—EFFECT OF HYPOPHYSECTOMY ON THE FEMALE REPRODUCTIVE ORGANS

No. of cat	Condition at operation	Time of killing after hypophy-sectomy, days	Ovaries		Average diameter of corpora lutea, mm	Weight of uterus
			Weight, gm	Condition		
CH 17	Probably immature	14	0·168	Small follicles	—	—
CH 46	Post-lactation	22	0·154	2 + 2 O.C.L.; S.F.	2·00	1·16
CH 52	Lactating	23	0·276	—	—	1·72
CH 39	Unknown	32	0·210	2 + 1 O.C.L.; P.F.	1·12	1·46
CH 14	,,	37	0·100	P.F.; C.F.	—	—
CH 11	,,	42	0·050	P.F.	—	—
CH 26	,,	51	0·319	2 O.C.L.; C.F.	1·12	1·90
CH 30	,,	52	0·158	4 ÷ 3 O.C.L.; S.F.	1·49	1·31
CH 25	,,	53	0·140	2 ÷ 2 O.C.L.; S.F.	1·48	3·35
CH 28	,,	65	0·108	1 − 3 O.C.L.; S.F.	0·82	0·99
CH 40	Pregnant	77	0·102	2 ÷ 1 O.C.L.; S.F.	0·62	1·42

O.C.L., Old corpora lutea. S.F., Follicles up to about 300 μ diameter showing the beginning of the antrum, but no larger normal follicles. P.F., Primordial follicles only. C.F., Cystic follicles.

recall Selye's (1933) observation that removal of the pituitary in the young rat does not necessarily lead to degenerative changes in the normal small follicles. The presence in the hypophysectomized cat of primordial follicles with fully grown ova, is in keeping with what has been found in other mammals, but not with the reaction in the fowl (Hill, R. T., and Parkes, 1934), in which hypophysectomy prevents the full development of the ovarian egg. The corpora lutea varied from quite large bodies, similar to the old corpora lutea of pregnancy in the normal lactating cat, to small fibrous corpora albicantia. None, however, were in a condition compatible with functional activity, and all showed obvious signs of atrophy such as the small size of the lutein cells and a high proportion of fibrous tissue, fig. 12, Plate 9. In CH 40, killed 77 days after hypophysectomy during pregnancy, the old corpora lutea of pregnancy were represented by very degenerate corpora albicantia, fig. 13, Plate 9. It is probable that at least this length of time is required for the corpora lutea of pregnancy to reach the same stage in the normal animal. Hypophysectomy of the cat has obviously not increased the morphological life of the existing corpora lutea as much as it is said to do in the rat (Smith, 1930; Selye, Collip and Thomson, 1933, *a*).

The uteri of the hypophysectomized cats were all smaller than normal uteri but, again, exact comparison is difficult because of the variation in the latter. Further, the weight alone gives an inadequate impression of the changes in the endometrium and muscular layers because the atrophy of the serous layer and connective tissue attachment is much less. In most of the hypophysectomized cats the myometrium, which is normally very extensive, was reduced to about half thickness. The endometrium was inactive and shrunken in all cases. Two types were observed. In the first, the lumen is narrow and the not very dense stroma is traversed by long inactive tubular glands. The lining epithelium is low columnar, fig. 14, Plate 9. CH 30, CH 46, CH 39, CH 25 and CH 28 had uteri of this type. The other type is more similar to that found in quiescent normal mammals. The endometrium is thin and dense, with a few atrophic glands, and the lumen is wide and contains masses of leucocytes and epithelial cells, fig. 15, Plate 9 (*cf.* lactating monkey uterus (Zuckerman, 1931) and anœstrous squirrel uterus (Deanesly and Parkes, 1933)). CH 40, CH 11, CH 14 and CH 26 had uteri of this type. The significance of the occurrence of these rather different types of uteri among the hypophysectomized cats is not apparent. The difference does not seem to be connected with the time after hypophysectomy or with the condition of the cat at operation. In any case it is evident that the ovarian atrophy leads to complete uterine inactivity.

No special features were observed in the vaginæ of the hypophy-sectomized cats. They were smaller than normal and the walls less convoluted. The epithelial lining in the lower vagina appeared very inactive and consisted of 2–3 layers of compact cells forming a basal membrane with 2–3 layers of squamous epithelium above. No trace was found of the tremendous thickening, stratification, and cornification which characterizes the normal vagina in the follicular phase of the cycle. In the upper vagina the epithelium was thinner, often being only one cell thick.

The Fallopian tubes were correspondingly inactive. They were shorter and straighter than normal; their walls were less convoluted and the folds less expansive. The epithelium was low cuboidal, as opposed to the tall columnar of the normal follicular phase, and the cells consisted mostly of nucleus so that there was no cytoplasmic border next the lumen. Cilia were absent in all the long-standing operated cats.

VI Effect on the Pregnant Female

There is some discrepancy in the results obtained by hypophysectomy during pregnancy in different species. Aschner (1912) early reported that abortion results in the dog, but in the rat (Pencharz and Long, 1931, 1933 and Selye, Collip and Thomson, 1933, a) and also in the mouse (Selye, Collip and Thomson, 1933, b) gestation may be pro-longed. In the guinea-pig hypophysectomy at 5 weeks leads to re-absorption, but at 6 weeks does not interfere with the normal course of gestation (Pencharz and Lyons, 1934). In the rat and mouse, where abortion follows double ovariectomy during pregnancy these results are extraordinary; in the guinea-pig where pregnancy may survive double ovariectomy in the last 3 weeks the results are more understandable. White (1932), on the other hand, has reported that abortion follows hypophysectomy of the pregnant rabbit.

As regards the cat, Allan and Wiles (1932) found that hypophysectomy in the later stages was followed by delivery of " normal-looking fœtuses," most of which were alive, at periods of 2-11 days after operation. Since, however, cat fœtuses are " alive " and normal-looking some time before normal parturition, and the pregnancies were undated, and the authors give no weights for the fœtuses at delivery, it is very difficult to say that this work shows that cat fœtuses are carried to normal term after hypo-physectomy. The authors specifically state that two litters were prema-ture. If the others were carried to term, then the operations were so near to term as to give little information about the effect of hypophysectomy

on the course of pregnancy. Allan and Wiles also record that no attempt was made to suckle live kittens, but they do not mention the condition of the mammary gland. The difficulty of obtaining cats at dated stages of pregnancy has prevented me from adding much to Allan and Wiles' data.

Four cats were hypophysectomized during the second half of pregnancy. CH 41 aborted five foetuses 2-4 days after operation. These foetuses weighed less than 20 gm each, and as the normal cat foetus at term weighs between 90-110 gm, there can be no doubt that this pregnancy was terminated at an early stage. The mother was killed after the abortion of the last foetus. The mammary gland, which was obviously that of a non-parous animal, had been developing well at the time of operation. Milk was not observed in the ducts. CH 42 aborted 4 days after operation and two foetuses were recovered. These weighed under 40 gm and were clearly premature. The mother was killed 8 days after operation. The uterus contained at least four obvious implantation sites so that presumably the other abortions had been eaten. The uterus appeared to be involuting normally. The mammary glands were also involuting, but abnormally, since the ducts were devoid of the milk which would have been present after an ordinary abortion, fig. 23, Plate 11. CH 37 was hypophysectomized, and 8 days later four apparently full-term young were aborted dead. Two mammary areas were removed by operation the following day; these were well developed, but showed no signs of milk secretion, fig. 22, Plate 11. The cat was then injected with anterior lobe extract and killed 6 days later. The remaining mammary glands had thickened greatly and milk was present in the ducts. CH 40 was a late pregnancy and probably an ex-lactator since milk could be expressed from the nipples at the time of operation. She gave birth to three kittens 2-3 days after hypophysectomy. These averaged slightly over 100 gm in weight and were clearly full term. Small drops of milk could still be expressed from the gland, but the kittens died in the course of 2-3 days. Owing to its condition at the time of operation the mammary gland of this cat gives no information as to the secretion of the gland after hypophysectomy, except that if adequate supplies of milk had been available the young might have been expected to survive, since the mother took care of them.

Control operations, consisting of exposing the pituitary, but not removing it, were performed on two cats in late pregnancy. One of these 6 days later had a normal litter of five which was suckled, while the second had six kittens of which four were born dead, but the other two were suckled. It is clear, therefore, that except possibly for the production of dead kittens by CH 37, the results described above must

be attributed to removal of the hypophysis rather than to the effects of manipulation.

In view of these findings and those of Selye, Collip and Thomson (1934), it seemed possible that results of interest might be obtained by removal of the pituitary during lactation. Two lactating cats were hypophysectomized, at 13 and 14 days post-partum respectively. The kittens of the first (Cat 74) lost weight rapidly after the operation, and by 7 days after the operation milk could not be expressed from the nipples. At 9 days the mother suddenly died and autopsy showed that the mammary gland, though still large, was quite dry. The second cat (CH 52) provides a more convincing example of cessation of lactation following hypophysectomy. In this case the kittens were fostered on to another lactating cat on the day of operation; on being returned the following day the mother " took " to them and suckling was continued. Seven days after operation the glands were drying off and it was difficult to express milk from the nipples. Subsequently no milk could be expressed. By this time the kittens were beginning to drink milk for themselves, but there is no doubt that this cat dried off much before it would have done in the ordinary way. When killed 23 days after operation, the glands that had been in use were still large but devoid of milk.

Atrophic mammary glands in the normal cat are sometimes faintly tinged with brown pigment. This was extremely marked in cats where an active gland had atrophied after hypophysectomy. CH 46 shows the phenomenon well. At the time of operation it was observed that she had obviously been suckling. Three glands (right inguinal and two abdominal) were marked as being in full secretory activity. Twenty-two days after operation, at autopsy, the glands presented a remarkable appearance, fig. 24, Plate 11. All the glands were atrophic, but the three which had been marked as active at the time of operation were a dark brown, while the others were the usual colour. Pigmentation to this extent is not found in the normal regressed gland, and it is presumably due to some aberration of the re-absorption mechanism following hypophysectomy. The photograph shown in fig. 24, Plate 11, was actually taken of the gland unstained, merely dehydrated and cleared in xylol. The glands which regressed before operation are clearly demarcated from the three regressing afterwards. The rate of atrophy of the gland after hypophysectomy, as indicated by the above experiment, is not dissimilar from that in the normal lactating cat after removal of the kittens, and there is no obvious reason why removal of the pituitary should cause a more rapid rate of regression than removal of the stimulus which causes the pituitary to maintain mammary secretion.

The ultimate state of the mammary gland of the hypophysectomized cat is such as might be expected after a long period of quiescence of the reproductive system (see mammary gland of Cat 40, fig. 25, Plate 11).

VII EFFECT ON THE MALE

Details of the three hypophysectomized males, compared with average data for six normal males, are given in Table IV. At 6 days after the operation little change has occurred in testis weight calculated on a body weight basis. At 18 and 47 days after operation progressive decrease has taken place and the testis is only about half its former proportion of body weight. This decrease is small compared with that which occurs in certain other species after hypophysectomy or even during anœstrus. The difference is probably due to the fact that the testes of the cat are always small in relation to body weight, as compared with animals such as the ferret, a fact which may be correlated with the absence of a period of quiescence in the male cat. In general, the maximum size attained by the testes is greater in animals with seasonal activity than in animals which are functional all the year round. It may be noted that the active ferret testis is about $0\cdot2$–$0\cdot3\%$ body weight and is reduced to about $0\cdot03$–$0\cdot04\%$ by hypophysectomy, whereas the active cat testis is only about $0\cdot06\%$ body weight, but is reduced by hypophysectomy to about the same as in the ferret, $0\cdot03\%$ body weight. For some reason, probably the high proportion of fibrous tissue, the size changes in the epididymis as shown by the figures in Table IV are not in keeping with the decrease in testis size.

Histologically the testis at 18 days shows considerable degeneration. There are no spermatozoa, few spermatids and the spermatocytes are reduced in number, fig. 16, Plate 10. At 47 days the ordinary picture of the atrophic testis of the hypophysectomized animal is seen. The tunica, always very thick in the cat, is further thickened by contraction. The tubules are small, Table IV, and consist only of basement membrane, spermatogonia, a few Sertoli cells, and a very occasional primary spermatocyte. There is no lumen, the tubules being occluded by syncytium. The intertubular tissue is increased in relative amount, though the interstitial cells are degenerate, fig. 17, Plate 10.

The epididymis shows the usual changes, including shrinkage of the tubule, with crinkling of the wall, and separation of the coils. The epithelium changes from columnar to dense cuboidal and the cilia disappear. At 18 days after hypophysectomy a few spermatozoa were still present, but at 47 days they had disappeared, figs. 18, 19, Plate 10.

TABLE IV—EFFECT OF HYPOPHYSECTOMY ON THE MALE REPRODUCTIVE ORGANS

| No. of animal | Time after hypophysectomy, days | Body weight at autopsy, kg | Testes | | | Weight of epididymides, gm |
			Weight, gm	Weight per kg body weight, gm	Average diameter 10 spermatic tubules, u	
CH 44	6	2·56	1·72	0·67	176	0·59
CH 50	18	2·00	0·89	0·44	95	0·29
CH 49	47	3·80	1·15	0·31	104	0·53
Average of six normals	—	3·55	2·31	0·65	180*	0·55

* Average of three normals.

Similar changes were observed in the vas deferens, figs. 20, 21, Plate 10.

VIII DISCUSSION

The work described above shows that hypophysectomy produces much the same effects in the cat as in other mammals. The most important of these are atrophy of the gonads and thence of the accessory reproductive organs, regression of the thyroids and adrenals, and disturbance of the carbohydrate metabolism characterized by increased sensitivity to insulin. As regards the last feature, the hypophysectomized cat is similar to the hypophysectomized dog, rabbit and monkey, but differs from the hypophysectomized ferret and fowl which do not show this increased sensitivity. The atrophy of the gonads apparently occurs in all species which have been hypophysectomized, and indicates that the pituitary has an essential function in the maintenance of gonad activity. The reaction with the adrenals seems to be more complex. Some regression clearly takes place after hypophysectomy and to this extent an adrenal-maintaining function may be ascribed to the pituitary, but this must be of an entirely different order of importance from the gonad-maintaining function. So far as gonad-function goes, hypophysectomy is equivalent to gonadectomy. But adrenalectomy is a lethal operation in cats whereas hypophysectomy is not, and hence hypophysectomy is not equivalent to adrenalectomy. The pituitary, therefore, does not apparently play the same essential part in maintaining adrenal function that it does in maintaining gonad function. This conclusion is in keeping

with the fact that it is much more difficult to demonstrate the adreno-tropic activity of pituitary extracts than to demonstrate gonadotropic activity.

IX SUMMARY

Cats have been hypophysectomized by a modification of the retro-pharyngeal route described by Allan and Wiles.

The general effects are similar to those described for other animals and include a greatly increased sensitivity to insulin. The thyroids and adrenals lose weight, and show histological regression, but since hypophysectomy in the cat is not a lethal operation, the adrenal atrophy is obviously not complete. The ovary, and thence the accessory organs, undergo atrophy and becomes quite inactive. No undue persistence of existing corpora lutea was noted.

Hypophysectomy in mid-pregnancy leads to abortion; hypophysectomy near term may be followed by the birth of living kittens, but these are not reared owing to lack of milk secretion. Hypophysectomy during lactation causes premature " drying off " of the mammary gland.

In the adult male, hypophysectomy causes atrophy of the testes and accessory organs similar to that occurring in other hypophysectomized mammals.

DESCRIPTION OF PLATES

PLATE 7

FIG. 4—Sella turcica of cat with pituitary *in situ* showing anterior lobe and, towards the dorsum sellæ, the posterior lobe surrounded by pars intermedia. × 15

FIG. 5—Sella turcica of CH 28 after removal of pituitary. Drill hole in basisphenoid nearly closed by regenerating bone. × 15

FIG. 6—Thyroid of normal cat showing large follicles and plump epithelial cells. × 150

FIG. 7—Thyroid of CH 39, 32 days after hypophysectomy, showing small follicles with flattened epithelium and large proportion of interfollicular tissue. × 150

PLATE 8

FIG. 8—Adrenal of normal cat showing development of cortex, particularly of the zona fasciculata. × 15

FIG. 9—Adrenal of CH 28, 65 days after hypophysectomy, showing reduction of cortex, particularly of the zona fasciculata. × 15

FIG. 10—Higher power of fig. 8, showing large vacuolated cells of zona fasciculata. × 150

FIG. 11—Higher power of fig. 9, showing decrease in size of fasciculata cells and grouping of nuclei. × 150

PLATE 9

FIG. 12—Ovary of CH 30, 52 days after hypophysectomy, showing degenerating corpora lutea and absence of follicles. × 11

FIG. 13—Ovary of CH 40, 77 days after hypophysectomy showing persistence of primary oöcytes, and two corpora albicantia which were corpora lutea of pregnancy at the time of operation. × 11

FIG. 14—Uterus of CH 30, showing decrease in the muscular coat and atrophic changes in the endometrium. × 16

FIG. 15—Uterus of CH 40, showing decrease in muscular coat and shrinkage of the endometrium. × 16

PLATE 10

FIG. 16—Testis of CH 50, 18 days after hypophysectomy. × 170

FIG. 17—Testis of CH 49, 47 days after hypophysectomy. × 130

FIG. 18—Epididymis of normal cat, showing presence of spermatozoa and active epithelium. × 70

FIG. 19—Epididymis of CH 49, showing absence of spermatozoa and degeneration and separation of the tubules. × 70

FIG. 20—Vas deferens of normal cat. × 70

FIG. 21—Vas deferens of CH 49, showing shrinkage of lumen and changes in the epithelium. × 70

PLATE 11

FIG. 22—Mammary gland of CH 37, removed by operation 9 days after hypophysectomy, abortion having occurred on previous day; a well-developed but non-secreting gland.

FIG. 23—Mammary gland of CH 42, 8 days after hypophysectomy and 4 days after abortion. Regression started but no milk secretion.

FIG. 24—Unstained preparation of mammary gland of CH 46 showing three heavily pigmented mammary areas and two unpigmented.

FIG. 25—Mammary gland of CH 40 at 77 days after hypophysectomy.

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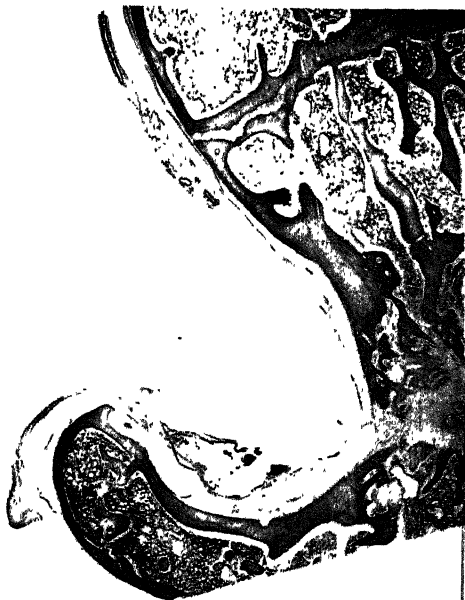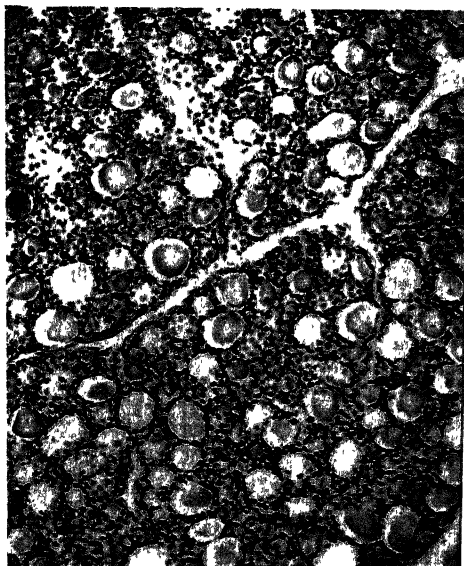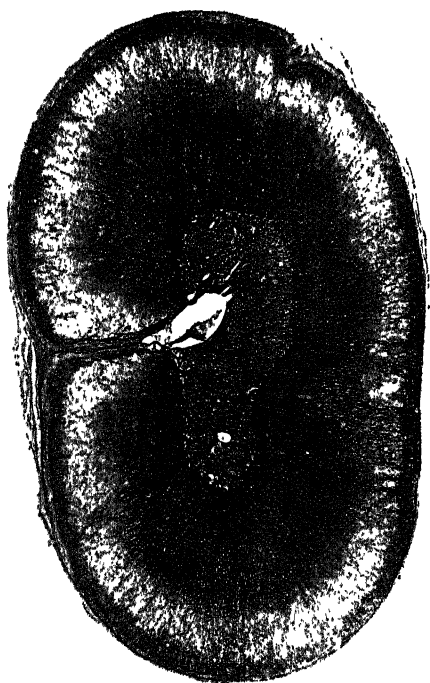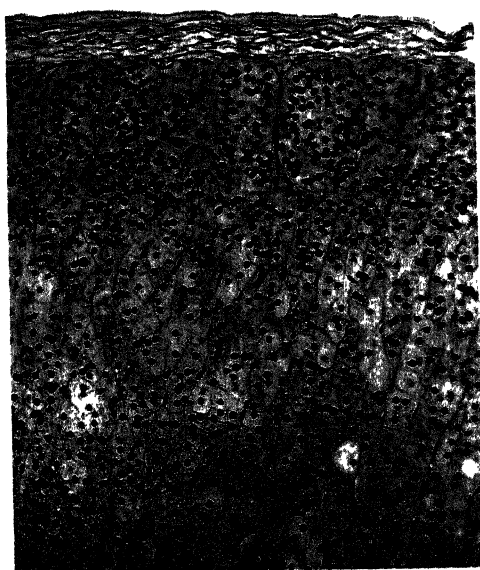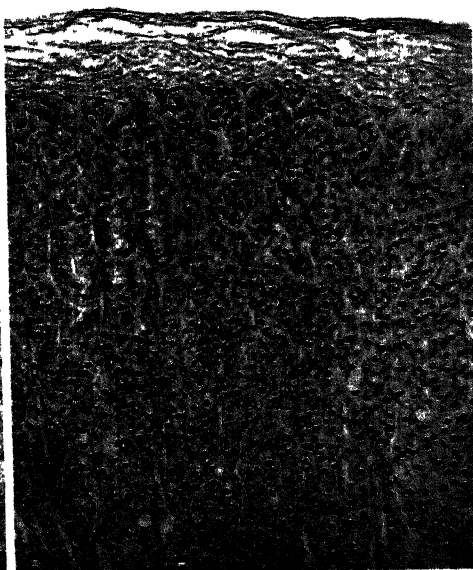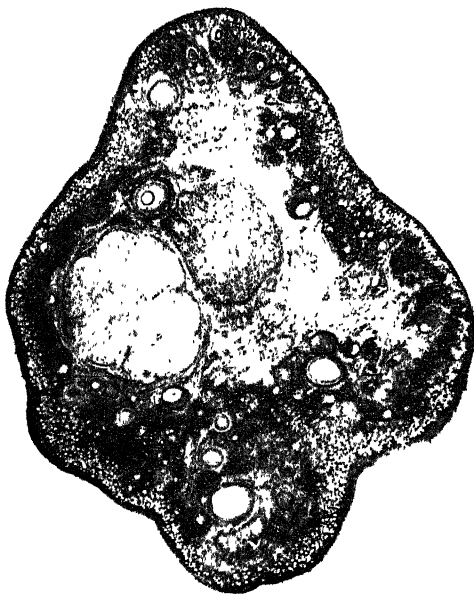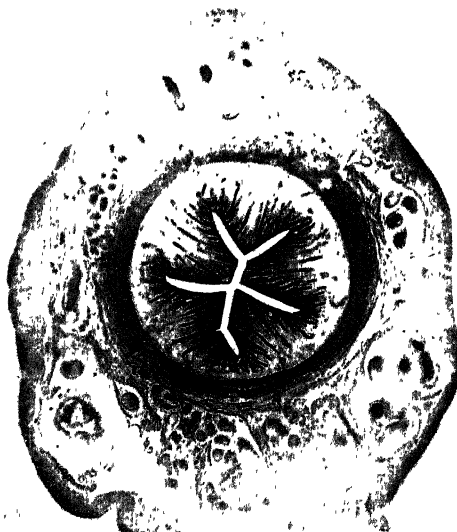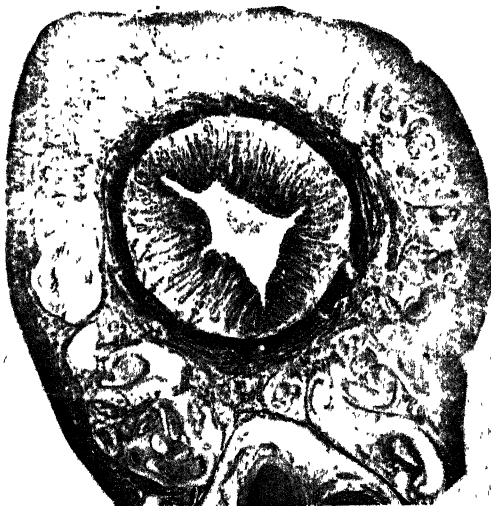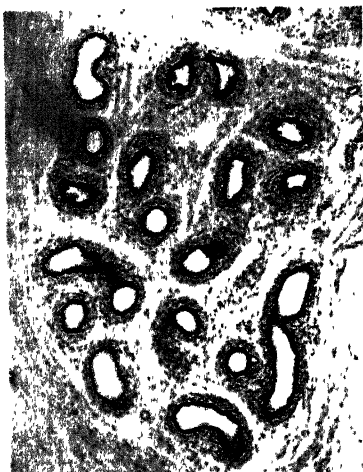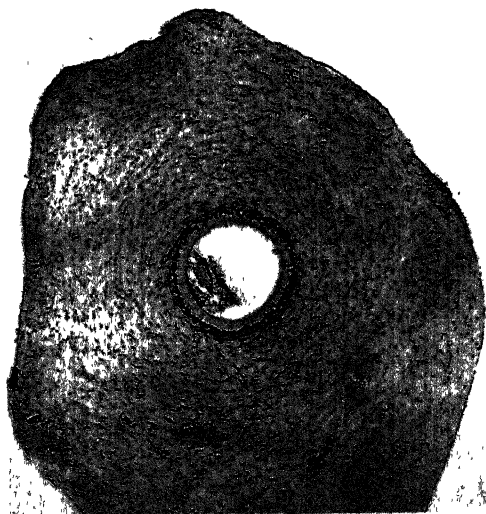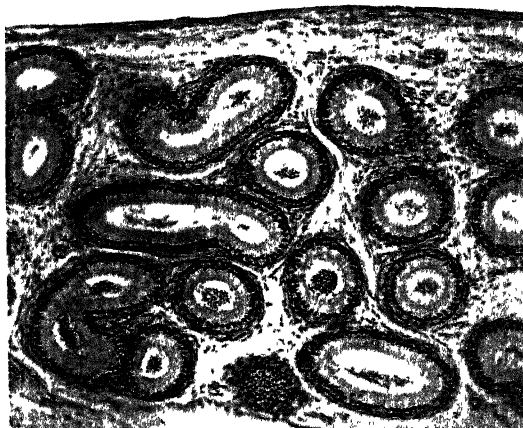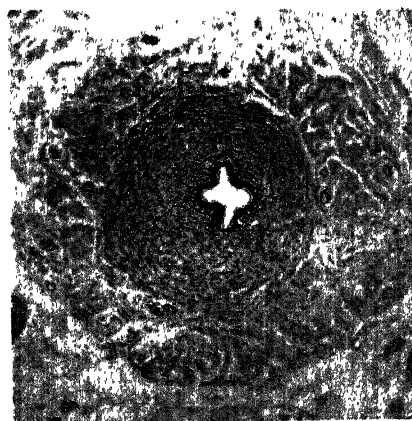
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The Detection of Linkage in Human Families

By Felix Bernstein with Richard Machol

In his papers Hogben (1934) deals with the product method of Bernstein (1929) to determine linkage in human families. Hogben, accepting the method, finds some faults in a part of the work of Bernstein and substitutes other formulæ and tables for those used by Bernstein for " indirekter Erkennbarkeit."

If the criticism were justified, the proof of the genetic independence of the old and the new blood groups given by Bernstein would be invalidated. The importance of this question justifies a detailed anwer. There are three objections:—

(1) That there is an error in the derivation of the formulæ for " indirekter Erkennbarkeit."

(2) That the case where one genotype is determinate and the other is not (p. 342) is not considered.

(3) That it was assumed that with one genotype determinate, the other not, the tables derived for the case where both the deter-

minate, are valid (p. 362).   Hence, the proof of the independence
of the old and new blood groups is not valid.

We shall consider these objections one at a time.

(1) All that needs to be said here is that Hogben obviously did not
know of the correction published by Bernstein (1932) which eliminates
this error, and which was first noticed and communicated to Bernstein
by Wiener.

(2) For this case, Bernstein made the claim that the formulæ for both
genotypes determinate hold true.   This will be further considered in
the reply to Hogben's final criticism.

(3) In reply to this, it must be said that Bernstein did not give proof
of his statement (of p. 136).   It was proved by the following derivation:
The work of the previous article holds good as far as the formulæ of
lines 16 and 17 of p. 128.   Here, as only one genotype is indeterminate,
we need use but one formula.   We choose (b)

$$\sum \frac{s!}{\alpha! \, \beta!} \left(\frac{p_1^\alpha p_2^\beta + p_2^\alpha p_1^\beta}{2} \right) = \tfrac{1}{2} [(p_1 + p_2)^s + (p_1 + p_2)^s] = (p_1 + p_2)^s,$$

therefore

$$w_A = 1 - (p_1 + p_2)^s$$

and

$$w_A (\mu^\kappa \nu^\iota)_{**}{}^0 = \sum \mu^\kappa \nu^\iota \frac{s!}{\mu! \, \nu!} \left(\frac{(p_1 + p_4)^\mu (p_2 + p_3)^\nu + (p_2 + p_3)^\mu (p_1 + p_4)^\nu}{2} \right)$$
$$- \tfrac{1}{2} \sum \alpha^\kappa \beta^\iota \frac{s!}{\alpha! \, \beta!} \, p_1^\alpha p_2^\beta - \tfrac{1}{2} \sum \alpha^\kappa \beta^\iota \frac{s!}{\alpha! \, \beta!} \, p_2^\alpha p_1^\beta.$$

We now go to the corrected article Bernstein (1932).

The formula of line 10 becomes:

$$w'_A (\mu^\kappa \nu^\iota)_{**}{}^0 = (\mu^\kappa \nu^\iota)^0 - (p_1 + p_2)^s (\alpha^\kappa \beta^\iota)^0$$

now

$$(\mu^\kappa \nu^\iota)^0 = s(s-1) pQ, \quad p = p_1 + p_4, \quad Q = p_2 + p_3$$

and

$$(\alpha^\kappa \beta^\iota)^0 = s(s-1) pQ, \quad p = \frac{p_1}{p_1 + p_2}, \quad Q = \frac{p_2}{p_1 + p_2}$$

and we get

$$w_A (\mu^\kappa \nu^\iota)_{**}{}^0 = [s(s-1)(p_1 + p_4)(p_2 + p_3)]$$
$$- \left[(p_1 + p_2)^s s(s-1) \left(\frac{p_1}{p_1 + p_2} \right) \left(\frac{p_2}{p_1 + p_2} \right) \right],$$

and

$$(\mu^\kappa \nu^\iota)_{**}{}^0 = \frac{s(s-1)(p_1 + p_4)(p_2 + p_3) - s(s-1)(p_1 + p_2)^{s-2} p_1 p_2}{w_A},$$

which gives

$$(\mu^x \nu^l)_{**}{}^0 = \frac{s(s-1)[(p_1+p_4)(p_2+p_3) - (p_1+p_2)^{s-2}p_1 p_2]}{1-(p_1+p_2)} .$$

Similarly the mean square deviation becomes

$$m_{**}{}^2(\mu\nu) = (\mu\nu)_{**}{}^0[(s-1)-(\mu\nu)_{**}{}^0]$$

$$+ \frac{s(s-1)(s-2)(s-3)}{w_A}[(p_1+p_4)^2(p_2+p_3)^2 - (p_1+p_2)^{s-4}p_1{}^2 p_2{}^2].$$

For the case of back-crossing (1) we have

$$p_1 = p_4 = \frac{1-c}{2}, \quad p_2 = p_3 = \frac{c}{2},$$

therefore

$$(\mu\nu)_{**}{}^0 = s(s-1)\frac{(1-c)(c)-(\tfrac{1}{2})^{s-2}\dfrac{(c-c^2)}{4}}{1-(\tfrac{1}{2})^s}$$

$$= s(s-1)\frac{c(1-c)(1-(\tfrac{1}{2})^s)}{1-(\tfrac{1}{2})^s} = \boxed{s(s-1)c(1-c)}$$

and

$$m_{**}{}^2(\mu\nu) = s(s-1)c(1-c)[(s-1)-s(s-1)c(1-c)]$$
$$+ s(s-1)(s-2)(s-3)[(1-c)^2 c^2]$$

$$= s(s-1)c(1-c)[(s-1)-s(s-1)c(1-c)$$
$$+ (s-2)(s-3)c(1-c)]$$

$$= \boxed{s(s-1)c(1-c)[(s-1)+c(1-c)(6-4s)]} ,$$

which are the same as the formulæ for back-crossing (1) with both genotypes determinate.

For the case of back-crossing (3) we have

$$p_1 = \tfrac{1}{2}-\frac{c}{4}, \quad p_2 = \tfrac{1}{4}+\frac{c}{4}, \quad p_3 = \frac{c}{4}, \quad p_4 = \tfrac{1}{4}-\frac{c}{4},$$

therefore

$$(\mu\nu)_{**}{}^0 = s(s-1)\frac{\left(\dfrac{3+4c-4c^2}{16}\right) - (\tfrac{3}{4})^{s-2}\left(\dfrac{2+c-c^2}{16}\right)}{1-(\tfrac{3}{4})^s} \qquad (1)$$

(Table II*b*)

and

$$m_{**}{}^2(\mu\nu) = (\mu\nu)_{**}{}^0[(s-1)-(\mu\nu)_{**}{}^0]$$

$$+ s(s-1)(s-2)(s-3)\frac{\left(\dfrac{16c^4-32c^3-8c^2+24c+9}{256}\right) - (\tfrac{3}{4})^{s-4}\left(\dfrac{c^4-2c^3-3c^2+4c+4}{256}\right)}{1-(\tfrac{3}{4})^s} .$$

(Table IV*b*)   (2)

However, when $c = \frac{1}{2}$

(1) becomes:

$$(\mu\nu)_{**}{}^0 = \frac{(\frac{1}{2})(\frac{1}{2}) - (\frac{3}{4})^{s-2}(\frac{0}{64})}{1 - (\frac{3}{4})^s} s(s-1) = \frac{\frac{1}{4} - \frac{1}{4}(\frac{3}{4})^s}{1 - (\frac{3}{4})^s} s(s-1) = \boxed{\frac{s(s-1)}{4}}$$

and (2) becomes:

$$m_{**}{}^2(\mu\nu) = \frac{s(s-1)}{4}\left[(s-1) - \frac{s(s-1)}{4}\right]$$

$$+ s(s-1)(s-2)(s-3)\frac{\left(\frac{1-4-2+12+9}{256}\right) - (\frac{3}{4})^{s-4}\left(\frac{\frac{1}{16}-\frac{1}{4}-\frac{3}{4}+2+4}{256}\right)}{1 - (\frac{3}{4})^s}$$

$$= \frac{s(s-1)(s-1)(4-s)}{16} + s(s-1)(s-2)(s-3)\frac{\frac{1}{16} - (\frac{3}{4})^{s-4}\left(\frac{81}{256\cdot16}\right)}{1 - (\frac{3}{4})^s}$$

$$= \frac{s(s-1)(s-1)(4-s)}{16} + \frac{s(s-1)(s-2)(s-3)}{16}$$

$$= \frac{s(s-1)}{16}\left[(s-1)(4-s) + (s-2)(s-3)\right] = \boxed{\frac{s(s-1)}{8}}.$$

These are exactly the same as the formulæ for back-crossing (3) with both genotypes determinate when $\frac{1}{2}$ is substituted for c. In the Landsteiner problem $\frac{1}{2}$ is the only value used by Bernstein for c, and hence his work and conclusions on that problem are numerically correct.

Therefore, it seems that Hogben has made an error in the derivation of his formulæ for this case in the crossing $AaBb \times Aabb$ (formulæ (26) and (27) on p. 359)[†] and therefore columns (iii) and (iv) in his Table I on p. 361 are incorrect so far as group (iii) of Table II (p. 361) is concerned, and this invalidates his final results.

Summary

The criticism of Hogben as far as it is justified, is met by a correction published by Bernstein before Hogben's article was submitted. The

[†] Hogben says (bottom p. 340) that Bernstein excludes the mating ($AaBb \times AaBb$) " on the grounds that the problem raised by" this mating "is only soluble when we know the relative frequency of the two possible types of parents having the constitution $AaBb$." Bernstein gave the formula for this case but remarked that such an hypothesis would increase the mean error and that this type is so rare in comparison with the others that it is not generally necessary to use it for the determination of linkage.

TABLE II*b*

For the probable value of $\mu\nu$ in the case of back-crossing (3)

$$\frac{AB}{ab} \times \frac{Ab}{ab} \text{ and } \frac{Ab}{aB} \times \frac{Ab}{ab}$$

by direct recognition of one heterozygote, and indirect recognition of the other.

$s =$	$c =$ 0·05	0·10	0·15	0·20	0·25	0·30	0·35	0·40	0·45	0·50
2	0·326	0·363	0·395	0·423	0·446	0·466	0·481	0·491	0·498	0·500
3	1·073	1·163	1·242	1·310	1·368	1·416	1·453	1·479	1·495	1·500
4	2·236	2·397	2·538	2·661	2·764	2·849	2·915	2·962	2·991	3·000
5	3·813	4·062	4·282	4·472	4·633	4·765	4·868	4·941	4·985	5·000
6	5·799	6·156	5·471	6·744	6·975	7·164	7·311	7·416	7·479	7·500
7	8·192	8·676	9·104	9·474	9·788	10·044	10·244	10·386	10·472	10·500
8	10·990	11·622	12·179	12·662	13·071	13·405	13·666	13·851	13·963	14·000
9	14·191	14·990	15·696	16·307	16·824	17·247	17·577	17·812	17·953	18·000
10	17·793	18·781	19·652	20·408	21·047	21·570	21·977	22·268	22·442	22·500

TABLE IV*b*

For the mean square deviation of $\mu\nu$ in the case of back-crossing (3)

$$\frac{AB}{ab} \times \frac{Ab}{ab} \text{ and } \frac{Ab}{ab} \times \frac{Ab}{ab}$$

by direct recognition of one heterozygote, and indirect recognition of the other.

$s =$	$c =$ 0·05	0·10	0·15	0·20	0·25	0·30	0·35	0·40	0·45	0·50
2	0·220	0·231	0·239	0·244	0·247	0·2488	0·2496	0·2499	0·249996	0·250
3	0·995	0·973	0·941	0·904	0·865	0·827	0·795	0·771	0·755	0·750
4	2·529	2·395	2·242	2·079	1·925	1·783	1·664	1·575	1·518	1·500
5	5·035	4·673	4·278	3·928	3·504	3·166	2·884	2·676	2·546	2·500
6	8·741	7·994	7·202	6·415	5·677	5·023	4·483	4·081	3·834	3·750
7	13·880	12·555	11·162	9·806	8·529	7·417	6·489	5·813	5·384	5·250
8	20·687	18·534	16·326	14·166	12·156	10·405	8·943	7·888	7·219	7·000
9	29·401	26·166	22·831	19·614	16·641	14·037	11·878	10·298	9·327	9·000
10	40·277	35·611	30·847	26·280	22·056	18·358	15·327	13·072	11·709	11·250

proof of the independence in the Landsteiner data was not invalidated, because as was pointed out, the tables based on the correct formulæ gave only very slight changes for the value of $c = \frac{1}{2}$, under consideration, so that a publication of the new valuation did not seem necessary.

The tables which Hogben substitutes differ widely and cannot be based on correct formulæ. Therefore his proof of independence is not valid.

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A Humoral Control of the Secretion of Brunner's Glands

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[PLATE 12]

It has been shown in a recent communication (Florey and Harding, 1934) that an alkaline mucin-containing secretion is obtained from fistulæ of that part of the duodenum containing Brunner's glands in several species of mammals. It is convenient to summarize here the evidence for believing that this secretion is that of Brunner's glands.

In the original communication on the subject (Florey and Harding, 1933) it was noted that an abundant flow of an alkaline mucoid secretion was obtained from a duodenal loop of a rabbit, anæsthetized with urethane, but that no fluid collected in an ileal loop. This observation has now been extended to fistula-bearing animals. Fistulæ of that part of the duodenum containing Brunner's glands continuously secrete an alkaline mucin-containing fluid (Florey and Harding, 1934). An ileal fistula of a rabbit or cat inserted by the same methods, does not secrete any juice which can be collected. Ileal fistulæ in the dog do not produce a

flow of succus entericus unless mechanically or chemically stimulated (Babkin, 1928, p. 770). Fistulæ of the proximal part of the duodenum, however, produce spontaneously a flow of mucoid juice amounting to as much as 1·5 cc an hour (Ponomarew, 1902; Florey and Harding, 1934). The striking histological difference between the duodenum and the ileum is the presence of Brunner's glands in the former. It is therefore reasonable to connect the flow of mucoid juice with these structures. This deduction is further strengthened by the fact that the cells composing Brunner's glands are of the type recognized as " mucous." Bensley (1903), in an extensive histological survey of the glands gives good evidence that they are mucus-producing organs, a view which our own observations support (Florey and Harding, 1934). Analyses of the juice in all species examined show that it contains either a mucin (goat, rabbit, sheep) or a substance very closely allied (cat, dog, pig). The source of this mucin must be either Brunner's glands or the goblet cells of the villi and crypts of Lieberkühn. The latter source is quite unlikely as it has been shown (Florey, 1930) that fistulæ of the colon in the dog do not secrete fluid but become plugged with inspissated mucus, yet in the colon goblet cells reach their maximal development. It has also been shown histologically (Florey and Harding, 1933) that Brunner's glands secrete when hydrochloric acid is perfused through the duodenal lumen and that a mucoid substance is added to the perfusing fluid. On the basis of all these observations, it is considered justifiable to conclude that the alkaline mucoid fluid collected from the upper part of the duodenum is, in fact, the secretion of Brunner's glands.

In certain species it has been noted (Florey and Harding, 1934) that the administration of food to an animal starved for 24 hours results in a well marked increase of the flow of juice from a duodenal fistula. Since the fistula is cut off from the main intestinal canal the stimulus to the glands in the fistula must be conveyed by nerves or by the blood stream. The following experiments have been directed to the solution of this problem.

<div align="center">EXPERIMENTAL</div>

The cat has been used almost exclusively for these experiments though for one purpose rabbits were employed. The juice secreted from the duodenal fistula of the cat sinks to zero after 24 hours starvation but on suitable feeding the secretion rate rises invariably to between 0·3 and 0·5 cc per hour. For the present experiments fistulæ were inserted in the way previously described (Florey and Harding, 1934).

Effects of Cutting the Vagi—Various procedures were adopted to obtain animals with a healed-in fistula and with both vagi cut. None, in which both vagi were first cut above the diaphragm and in which about a fortnight later a fistula was inserted, have given good results. A satisfactory preparation, however, was made as follows: at a first operation, through a mid-line abdominal incision, the left vagus was cut above the diaphragm, intratracheal ether anæsthesia being used. After section of the nerve the abdomen was closed and allowed to heal. At a second operation a duodenal fistula was inserted in the usual way. When this had healed readings of the rate of flow and the influence of food intake were made; these revealed the reactions to food obtained in a partially innervated fistula. At a third operation the right vagus was cut in the neck. Thus by the two nerve section operations the abdomen was completely removed from the influence of the vagi. Readings of the rate of the flow, etc., were then repeated.

Results—With both vagi cut.

Animal starved for 24 hours, being allowed water only, before commencement of collection—

        1st hour .... 0·0 cc
        2nd hour.................. 0·0 cc

After feeding with meat and milk—

        1st hour .... 0·2 cc
        2nd hour.................. 0·4 cc
        3rd hour .... 0·4 cc

At a post-mortem examination a dissection showed complete section of the left vagus, on the central end of which was a knob-like neuroma. The right vagus was cut in the neck and all communicating branches between the two vagi had had their paths to the abdomen interrupted.

It is thus clear that the administration of food, after a starvation period, produces a flow of juice independently of any vagal impulses.

Effect of Cutting the Splanchnic Nerves—In the experiments to be quoted different procedures were adopted. In one animal the fistula was inserted at a first operation and, after healing was complete, the splanchnic nerves were cut through loin incisions. With the nerves cut and healing complete the following figures were obtained:

Animal starved 24 hours—

       1st hour .... 0·0 cc secretion

       2nd hour................. 0·0 cc   ,,

       3rd hour .... 0·0 cc   ,,

Animal then fed, meat and milk—

       1st hour .... 0·3 cc secretion

       2nd hour................. 0·4 cc   ,,

       3rd hour .... 0·3 cc   ,,   (some leaked into bandage)

       4th hour .... Pot slipped

       5th hour .... 0·5 cc secretion

A similar result was obtained in a subsequent experiment.

In another animal the right splanchnic nerve was first cut in the chest under intratracheal ether. When healing was complete the left splanchnic nerve was cut through a midline incision and a fistula inserted. The following figures were obtained from this animal:

Animal starved 24 hours—

       1st hour .... 0·0 cc secretion

       2nd hour................. 0·0 cc   ,,

       3rd hour .... 0·0 cc   ,,

Animal then fed, meat and milk—

       1st hour .... 0·2 cc secretion

       2nd hour................. 0·4 cc   ,,

       3rd hour .... 0·4 cc   ,,

Unfortunately it was found at post-mortem examination that fine accessory splanchnics were present in both animals and that these remained uncut. No readings were obtained from a third cat in which both the main and accessory splanchnics were cut owing to the premature death of the cat, but it was quite clear that the fistula of this cat secreted normal looking juice.

These experiments allow of the deduction that impulses conveyed by the great splanchnics are not the cause of the response to the ingestion of food. Probably the lesser splanchnics are also not involved. These

experiments were not followed further as it seemed necessary to effect a total extirpation of the abdominal sympathetic chains as well as division of the splanchnics to be sure of the result, but the information derived from the above experiments has been substantiated in another way which gives clear-cut results.

The Effects of Atropine and Pilocarpine—Pilocarpine injected intravenously causes evacuation of the mucin of Brunner's glands with the production of a mucoid alkaline fluid similar to that produced from fistulæ (Florey and Harding, 1934).

In acute experiments atropine was found to have no influence on the secretion caused by passing HCl over the upper duodenal mucosa.

Experiments have now been done on the effects of atropine on the secretion from a fistula following food. It has been found that the injection of 3 mg of atropine subcutis has no influence on the rate of secretion though this dose was sufficient to paralyse the pupil reflex to light.

Auto-transplantation of the Duodenum—Ivy and Farrell (1925) introduced a procedure which they called auto-transplantation. The principle involved is as follows: an abdominal organ or portion thereof is transplanted into the subcutaneous tissues, the blood supply being maintained through a pedicle containing the abdominal nerves and vessels. After a period of healing the pedicle is cut at a second operation. A certain amount of collateral circulation now exists between the skin vessels and the abdominal organ. If this is sufficient the organ lives and any stimulation reaching the organ must now come through the skin blood supply.

Our first experiments were done with rabbits as, in this animal, the lower duodenum is supplied with a free mesentery.

A fistula, made as previously described (Florey and Harding, 1934), was inserted subcutis in one animal and in another between the layers of the abdominal muscles.

In the latter animal the fistula was allowed to heal in for 29 days and then at a second operation the pedicle was cut. The pedicle was greatly elongated and appeared as a rounded cord. The anastomoses with the muscular vessels were not adequate to carry on completely the nutrition of the isolated duodenum as some sloughing occurred. After 21 days however, regeneration of Brunner's glands had occurred and the usual mucoid secretion was again obtained. A timed collection was made 33 days after cutting the pedicle with the following results:—

1st hour .... 0·5 cc secretion
2nd hour.................. 0·5 cc   „
3rd hour .... 0·3 cc   „   juice soaked
                                        into bandage
4th hour .... Pot slipped
5th hour .... 0·5 cc secretion

The juice was clear and mucoid and indistinguishable from that of an innervated fistula. Mucin was precipitated from it by acetic acid.

In the other animal, 43 days after the original operation the elongated pedicle was cut before the ligature was tied and only a few drops of blood oozed from the central end. The fistula was thus already being supported by the collateral skin circulation. As was to be expected no sloughing occurred in this animal and secretion proceeded quite normally. This isolated fistula produced juice at the rate of 0·4 cc an hour.

Post-mortem and Histological Examinations—Both animals were killed to enable an examination of the conditions present to be made. The first animal was killed 62 days after the pedicle had been cut. It was found that the fistula was totally isolated in the belly wall without any connection with the rest of the viscera. Histologically it was seen that Brunner's glands had regenerated and were in fact still regenerating. Not only did the glands occupy a position below the muscularis mucosæ but they had grown into the place formerly occupied by the crypts of Lieberkühn. The villi had disappeared and the gut surface was covered with columnar epithelium among which goblet cells were scattered. It was found that the fistula in the second rabbit, killed 61 days after the original operation, was also completely isolated in the belly wall. Histologically the gut structure was almost perfectly retained. A comparison of the two microphotographs from this animal shows this better than any description, figs. 1 and 2, Plate 12. Unfortunately the rabbit is an animal in which it has not been possible to show the effect of the ingestion of food on the rate of flow of juice, probably owing to the difficulty of obtaining an empty stomach. Nevertheless, these experiments clearly showed that secretion of an alkaline mucoid fluid, physically indistinguishable from that obtained from an innervated fistula, was secreted by a totally denervated and isolated fistula. The presumption was strong that some humoral influence, either of the nature of a hormone or a secretagogue was involved in the process. It was thus necessary to extend these experiments to the cat.

The technique of the operation was the same as that previously described except that the fistula was completely pulled out through the mid-line and the skin sewn together over it. The exit of the fistula was made through a small buttonhole of the skin to one side of the mid-line. Considerable tension existed on the pedicle of a fistula so prepared. In one successful preparation the pedicle, which was broad and thick, was cut on the 41st day after the original operation, so that at the end of the second operation no connection remained between the fistula in the abdominal wall and the rest of the abdominal contents. At the conclusion of the operation the mouth of the fistula was a pink colour and obviously had a good blood supply from the skin. In this animal there was no sign of sloughing of the fistula after cutting off its abdominal blood supply. The animal continued to eat well and the day after the operation juice was noted coming from the fistula following food. During the first week abundant juice, which was soaked up by the dressings, was secreted but no measurements were made owing to the difficulty of applying a pot in the presence of the recent mid-line incision.

The animal was starved for 24 hours on the 10th day after cutting the pedicle and the following figures were obtained next day:—

Animal starved 24 hours—

1st hour	0·0 cc	secretion
2nd hour....................	0·0 cc	„

Fistula was quite dry.

Then fed meat and milk—

1st hour	0·3 cc	secretion
2nd hour..................	0·5 cc	„
3rd hour	0·5 cc	„

The secretion was clear and mucoid as from an innervated fistula.

On the 18th day the procedure was repeated with identical numerical results.

The animal was killed on the 25th day after cutting the pedicle, to enable an examination to be made. This revealed that the omentum had become adherent along the mid-line wound. It was thus possible that the omental vessels in the later stages of the experiment contributed some blood for the maintenance of the subcutaneous fistula. Nevertheless it was certain that at the pedicle-cutting operation all nervous and vascular connections were severed between the fistula and the abdominal contents. Secretion from the fistula was noted from the day after the operation

onwards, that is, before any omental adhesions carrying a blood supply could be effective. Whether the omental adhesions did eventually carry a blood supply or not is really immaterial to the interpretation of the experiment, which shows clearly that, after complete section of all abdominal nerves to the fistula, secretion continued and showed the usual quantitative responses to starvation and food which have been invariably found in innervated fistulæ. Histological examination of the transplanted fistula showed that both Brunner's glands and the rest of the mucosa had been preserved intact in spite of the alteration of the source of blood supply, figs. 3 and 4, Plate 12.

In another successful preparation the pedicle was cut 40 days after the insertion of the fistula. Again at the end of the operation no connection existed between the fistula beneath the skin and the abdominal contents. A few hours after the operation the cat took food freely and secretion in considerable quantity was seen issuing from the fistula. On the second day after the operation an hourly collection was made. The rate was found to be 0·3 cc per hour—a figure exactly the same as that before the operation. After 24 hours starvation a collection was made on the fourth day after operation. Two hourly samples showed no secretion. Meat and milk were then given and the rate rose to 0·3 cc per hour. The cat was killed on the 9th day after cutting the pedicle to enable an examination to be made. This revealed that the fistula was totally isolated beneath the skin from which it obtained its whole blood supply as no omental adhesions were present anywhere near the fistula. Only one small adhesion of the omentum was present at the lowest point of the mid-line abdominal wound about 6 cm from the fistula.

Amount of Bicarbonate present in Juice from Fistulæ of Cat—Many determinations on 12 cats of the amount of bicarbonate present in Brunner's gland secretion have been made. The figures obtained were usually 1·0 cc secretion equivalent to between 0·25 and 0·35 cc N/10 soda. It will be noted that these figures are lower than those given previously which were obtained on one cat (Florey and Harding, 1934). They are about the same as those found for the dog. No striking effect on the bicarbonate content of the juice was noted after total denervation; *e.g.*, in one cat before cutting the pedicle two collections gave 1·0 cc juice equal to 0·25 cc and 0·35 cc N/10 soda respectively. After cutting the pedicle 1·0 cc juice was equal to 0·25 cc N/10 soda. In another cat the figures before and after were respectively 1·0 cc juice equal to 0·38 cc and 0·31 cc N/10 soda. In the rabbit also 1·0 cc juice equalled 0·66 cc N/10 soda before cutting the pedicle, while afterwards 1·0 cc juice equalled 0·55 cc N/10 soda.

DISCUSSION

The results obtained by the section of the vagi and of the splanchnic nerves suggested that the stimulating factor involved in Brunner's gland secretion was blood borne. That this was the case was shown by the experiments in which the duodenum was transplanted into the abdominal wall with all nervous connections cut. Fistulæ of this kind can only secrete when activated by a substance carried by the skin vessels. Whether the substance involved is a hormone or something absorbed during digestion is at present being investigated.

It is important, however, to realize that these results have been obtained on the cat in which secretion stops after 24 hours starvation. In the dog and other species, such a period of deprivation was not sufficient to reduce the flow to zero, although it was considerably reduced from the amount secreted after feeding (Florey and Harding, 1934). The possibility therefore exists that a continuous flow is maintained by some mechanism but that the administration of food causes the production of a secretagogue or hormone to increase the steady flow. The mechanism maintaining the steady flow may conceivably be of nervous origin.

The effects of pilocarpine may be thought to indicate that the glands are under the influence of the parasympathetic system, but with increasing knowledge it becomes apparent that the effects it produces have not necessarily any connection with innervation. Moreover, the inability of atropine to inhibit the secretion is against the active participation of the vagus.

SUMMARY

The secretion of Brunner's glands of the cat and rabbit occurs independently of extrinsic innervation. The glands of the cat are activated after the taking of food by a blood-borne stimulus—a hormone or secretagogue.

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FIG. 1
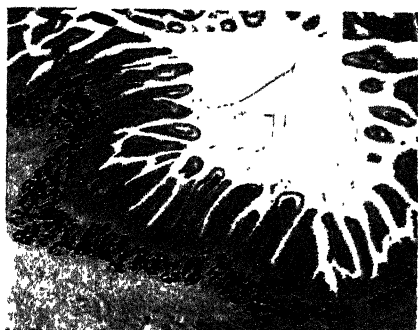
FIG. 2

FIG. 3
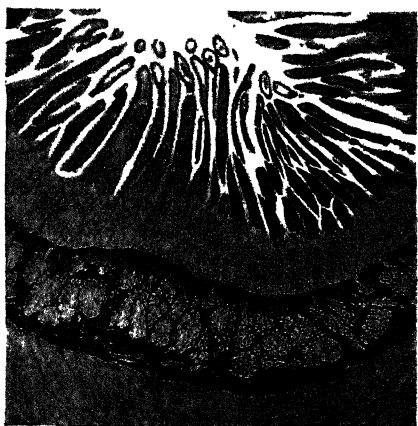
FIG. 4
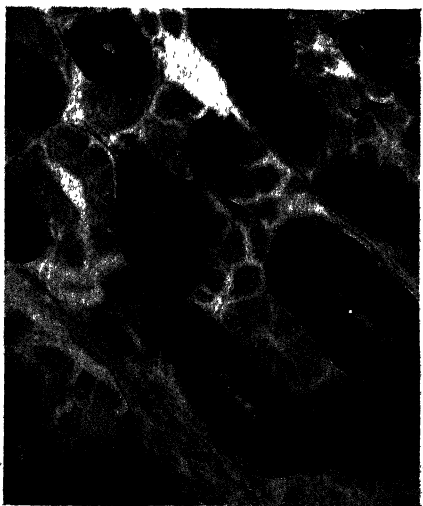
FIG. 5
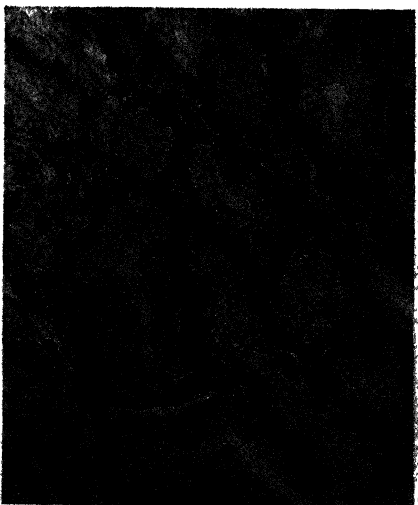
FIG. 6

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DESCRIPTION OF PLATE 12

FIG. 1—Rabbit. Duodenum near anastomosis. Fixed in Helly's fluid, stained hæmalum, mucicarmine and metanil yellow. Shows the normal appearance of Brunner's glands in the lower part of the duodenum. Brunner's glands are stained intensely red which appears black in photograph. × 17·5.

FIG. 2—Rabbit. Same rabbit as fig. 1. Subcutaneous transplant of piece of lower end of duodenum. Same histological procedure as for fig. 1. Shows the preservation of Brunner's glands and of the structure of the villi. × 17·5.

FIG. 3.—Cat. Normal duodenum to show layer of Brunner's glands. Fixed in Helly's fluid, stained by the azan method. × 17·5.

FIG. 4—Cat. Subcutaneous transplant of duodenum. Same histological technique as for fig. 3. Shows the preservation of Brunner's glands as well as the rest of the intestinal structures. × 17·5.

FIG. 5—Rabbit. A portion of Brunner's glands from preparation of fig. 2. Shows the mucus-containing cells (black) and the "serous alveoli" which are peculiar to this species. Note the perfect preservation of normal cellular structure. × 437.

FIG. 6—Cat. A portion of Brunner's glands from the preparation of fig. 4. Shows the mucus-containing cells which are indistinguishable from those of a normal duodenum. × 437.

The Experimental Induction of Melanism, and other Effects, in the Geometrid Moth *Selenia Bilunaria* esp.

By J. W. Heslop Harrison, D.Sc., F.R.S.

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[Plate 13]

1—Introduction

In the autumn of 1927, Hughes, wishful to repeat the work of Harrison and Garrett (1926) on the induction of melanism in the Lepidoptera, visited my laboratory to consult me about material and methods. As a result of the information supplied, his investigations were initiated in the spring of 1928. Very soon, however, he informed me that his progress was being materially hampered by the fact that the broods with which he was dealing were " full of lethal factors." This complaint he repeated with still greater emphasis when the important work with treated larvæ was proceeding.

Now *Selenia bilunaria* has been a favourite experimental subject of mine for more than 25 years, and, further, my wild broods have been derived from no less than forty-seven different localities in England, Scotland, Wales, Ireland and Germany; in spite of these wide opportunities, never at any time have I encountered trouble in rearing large broods, nor have any difficulties ever arisen which could be interpreted by appeal to lethal factors. Hence I was forced to suspect that Hughes' cultures were probably infected with polyhedral disease (due to a filterable virus) or with others, sporozoan or bacterial in their origin.

2—Experiments Designed to Induce Melanism

Influenced by the early results of Hughes' researches and by his statements, I determined once more to attempt the induction of melanism in *Selenia bilunaria,* and also to test the effects of the introduction of one of the diseases to which reference has just been made, into cultures carrying melanism.

A batch of eggs, 227 in number, was therefore obtained from Thuringia in the spring of 1930. It gave rise to 211 larvæ which were reared on untreated hawthorn at Birtley, Co. Durham. During their early instars, they were kept in the usual glass-topped tin box. These were sub-·stituted in the later stages by cages specially designed to secure a maximum of fresh air and a minimum of unnecessary moisture.

In the brood (Family A) which appeared in the following July* 189 imagines, 98 males and 91 females were included. From these insects one pair was caged up, and the larvæ resulting subjected to a diet of hawthorn sprayed with $0 \cdot 1\%$ solution of manganous sulphate. At this stage, as will be seen from Tables I and II, the methods of control adopted in the earlier work were drastically altered. Instead of inbreeding part of the original stock, I took half a dozen other insects from family A and outcrossed them with melanics descended from family *d* of the 1928 paper. Of the larvæ so derived, fifty were chosen from each mating and reared on pure hawthorn.

Under such circumstances, had any of the parents taken from family A been heterozygous for melanism, certain of these control batches ought to have developed melanics; only types, however, appeared.

In the next generation the treated batch was inbred, and again the offspring of a single pair reserved for treatment. On the other hand, once more six of the brothers and sisters of that pair were mated with melanics of the same origin as the previous lot, and fifty larvæ from each control batch reared. This procedure was kept up with the successive generations until July, 1932.

The outcome of all these matings, comprising both control and treated lots, with all the necessary information about the pigmentation of the imagines, is set out in Tables I and II.

TABLE I—TREATED BROODS

Family	Origin of family	Number of eggs	Females		Males	
			Types	Melanics	Types	Melanics
M′	Ex family A	182	71	—	83	—
M²	M′♀ × M′♂	202	73	—	87	—
M³	M²♀ × M²♂	169	59	—	64	—
M⁴	M³♀ × M³♂	196	61	—	72	—
M⁵	M⁴♀ × M⁴♂	149	65	1	60	—
	Totals	898	329	1	366	0

* I have no longer facilities for rearing more than two broods in the year with safety.

TABLE II—CONTROL BROODS

Families	Origin of families	Females		Males	
		Types	Melanics	Types	Melanics
Control batches 1, 2, 3, 4, 5, 6, totalled	All A × d	119	—	129	—
Control batches 7, 8, 9, 10, 11, 12, totalled ..	All M′ × d	111	—	122	—
Control batches 13, 14, 15, 16, 17, 18, totalled	All M² × d	102	—	113	—
Control batches 19, 20, 21, 22, 23, 24, totalled	All M³ × d	116	—	138	—
Control batches 25, 26, 27, 28, 29, totalled ..	All M⁴ × d	115	—	109	—
Control batch 30	M⁴ × d	19	1	22	—
Totals		582	1	633	0

As will be detected at once, for four generations no melanics appeared, either amongst the treated batches or the controls. In the fifth generation, however, both controls and treated broods gave rise to melanics for one* control family and treated family M_5 each contained a single melanic female, fig. 1, Plate 13. Unfortunately, the single melanic female developed in the treated section did not emerge until the first week in May when the eclosion of males had long since ceased. Nevertheless, several typical males, kept alive for a fortnight in case a melanic emerged, were caged with her; no pairing took place owing to the age of the males.

The mode of inheritance of the induced melanism could not, therefore, be tested, but there are no reasons for thinking that it would have varied from that studied in the 1926 and 1928 experiments.

At this point the sporozoan disease, deliberately introduced to certain *Selenia* cultures for purposes described below, became so virulent that, to prevent the infection of certain critical *Oporinia* broods, all of the *Selenia* material was discarded.

3—EXPERIMENTS RESULTING IN THE DEVELOPMENT OF ALBINISTIC INSECTS

These experiments were not planned deliberately to bring about the induction of albinism for they were undertaken quite casually in October, 1931, when I found it impossible to supply wild larvæ of *Selenia bilunaria,*

* The significance of this occurrence is discussed later.

collected from raspberry (*Rubus Idæus*), with regular supplies of their natural food; they were consequently transferred to alder (*Alnus rotundifolia*). This was accepted with avidity, but in its turn, owing to its progressive deterioration and the approach of winter, it failed before the larvæ could spin. Naturally, most of them died, but five changed successfully to pupæ, three of which were females and two males— somewhat remarkable figures for a starved brood. Probably, however, they owe their origin to the circumstance that female larvæ feed longer than males, and consequently later beaten batches include a preponderance of females.

These pupæ were remarkable in respect both to size and colour. In size, four of the pupæ (2 females and 2 males) approximated to that proper to *Melanippe sociata* whilst the third female was of the same size as a pupa of *Melanippe tristata*. The colour, black in normal, full-sized pupæ, was of a pale clear yellow, recalling the pupæ of the Geometrid *Acidalia herbariata*, or the Noctuid *Miselia oxyacanthæ*.

Very little hope was entertained of their wintering safely, but, in spite of that, they all emerged, practically simultaneously, in April, 1932. Two of the females were mated with the males and after pairing they were removed from the cages and put aside in chip boxes to lay. The third female was then placed in the cage: it likewise copulated with one of the males and deposited a small batch of eggs.

As I have already indicated in previous papers, *Selenia bilunaria* is strongly seasonally and sexually dimorphic, with the spring emergence consisting of large, heavily marked, richly pigmented insects, and the summer brood of paler and more lightly marked specimens, fig. 1, Plate 13. With the present insects, fig. 1, however, the two sexes, although appertaining to the spring generation, display no dimorphism and reveal themselves as tiny creatures even lighter in hue and more feebly marked than the normal summer male.

Of their eggs, only 29 emerged, and the larvæ, in colour and pattern pale, sandy, washed-out looking animals, were treated as a mass culture. For food they were given the cleanest and best hawthorn procurable; in rearing them, too, every possible care was taken. They yielded 21 pupæ of which the chitin was exactly the same colour as in the parents.

In June, 21 imagines were bred which, despite all the attention bestowed upon the larvæ, were only a little larger than their parents. No seasonal dimorphism was displayed, and the insects, save for one albinistic male, were, to all intents and purposes, replicas of their parents, and hence lighter than *juliaria* forms. In the normal form, the forewings possess a pale brown ground colour, sprinkled with blackish scales. Across them

pass three narrow, dark ferruginous lines, and at the apex is a ferruginous patch. In the hind wings, the ground colour is the same, but only the median line appears. The thorax and abdomen are of the same pale brown.

On the other hand, in the aberrant example the ground colour of the wings and of the abdomen is of a clear silvery white, whilst the wing markings, of a very light sandy colour, are obsolescent and the blackish sprinkling entirely absent.

The majority were caged together once more to give a mass culture, whilst the albino was mated with one of its sisters (family T). Ova duly appeared in both cages, and most hatched. Once again extreme care was taken to supply the larvæ with the best of food and attention. The larvæ, however, were still pale rust in colour, but the pupæ were, perhaps, just a little darker than those of the preceding generation.

With very little loss, both batches yielded their moths in spring 1932. Again, in family U, the moths were paler and much smaller than those of an ordinary summer brood, and thus very much inferior in those respects to normal spring insects. Still, a little improvement in size and colour could be discerned.

TABLE III—DESCENDANTS OF STARVED INSECTS

Family	Origin of family	Females	Males
S (wild lot)	Beaten larvæ	3	2
T	S♀ × S♂	7	14
			(1 albino)
U	T♀ × T♂	5	13
U′	T♀ × albino ♂	3	5
V	U♀ × U♂	5	8
W	V♀ × V♂	6	9
Totals............		29	51

Family U′, resulting from the mating between the albino and its more ordinary sister, had precisely the same facies, so that, if the albinism had been inherited, it had behaved as a Mendelian recessive.

Both families U′ and U were inbred; unfortunately the brood from U′ failed to respond to all the attention it received and died out; thus the critical F_2 lot necessary for demonstrating that the albinism had been inherited was not procured. On the other hand, the inbreeding of family U put me in possession of pupæ a little more robust than the

previous batch and finally imagines, also a trifle larger and a little more decidedly marked (family V).

Inbred once again, family V yielded a small number of pupæ, yellow in colour, and moths (the vernal brood, of course) slightly larger but still vastly inferior in every respect to the usual spring moths, and even far from approaching in appearance the summer generation *juliaria*. Moreover, no hint was offered of sexual dimorphism (family W).

An attempt was made to carry the experiment further, but without success. Most of the ova deposited were unfertile, and the larvæ which hatched failed to feed up. Table III embodies all the statistics derived from these experiments; their import and that of other pertinent facts are discussed in the concluding remarks.

4—Concluding Remarks

Although these researches were undertaken chiefly from the desire to ascertain the causes of Hughes' difficulties, and his final failure to induce melanism in *Selenia bilunaria*, advantage is taken of the opportunity for reviewing the labours of other investigators (Thomsen and Lemcke, 1932, and Walther, 1932), and the criticisms of Sinnott and Dunn (1925), Muller (1929) and Sonneborn (1930). Further, it is to be understood that, in any comparison of my cultures with those of others, no reference is made to the facts tabulated in the 1926 paper for two reasons: (1) that paper lists certain broods over which I had no control, and (2) the treated batches it describes were not whole broods but portions purposely limited.

The Experiments of Hughes—Hughes, in the presentation of his results, throws the bulk of them into the form of long tables, very impressive from their size, but actually of little value as proof or disproof of the validity of my 1928 work. In the first place, they are so set out that one is almost compelled to attach equal importance to all treated broods labelled, for example, as belonging to the fifth generation, whereas the actual facts are that that group may, and often does, include broods whose ancestors have received treatment restricted to one generation. Secondly, no cultures are described which had partaken of lead or manganese contaminated food for more than four generations; thus, they cannot, with complete justice, be used in any criticism of cultures in which success was obtained in the fifth generation of treatment. Even granting that a positive result might, on the basis of the 1926 work of Harrison and Garrett, have been legitimately anticipated, the sizes of the

families reared in the final generations appear quite inadequate for critical work.

In the lead series, the fourth treated generation included 177 insects, but these belonged to no fewer than 26 families, an average of 6·8 a family, whilst the fourth manganese lot comprised two families only with 13 moths, an average of 6·5.

This failure to rear broods of substantial size forms the outstanding feature of Hughes' researches, and it is proposed now to look into the cause or causes thereof and to focus attention on their probable effects.

Hughes makes the unequivocal statement that in only one instance was disease seen; yet, on analysing his tables closely the following extraordinary series of facts emerges :—

(1) No fewer than 30 broods are accompanied by the remark (or one similar) that they " Died."

(2) Seven are described by the word " Failed."

(3) The colossal number of 225 matings is also labelled " Failed."

If 30 broods died, some reason must be assigned for the occurrence, for lepidopterous larvæ, more especially those of such an unusually easy subject as *Selenia bilunaria*, do not die on such a scale without adequate cause. In my long experience, involving many hundreds of broods, no parallel mishaps have befallen my larvæ. Unless, therefore, neglect or their deliberate destruction is the cause, disease of some type must be invoked. A similar remark applies to the broods which " failed."

But what can be said about the 227 matings which likewise " failed " ? During the course of my experiments with *S. bilunaria* thousands of healthy pairs from 47 different localities, and many combinations of these, have been caged up; yet never, as far as I have records or can remember, has one failed. However, it can be put forward, that moths reared from diseased larvæ often refuse to pair, and, even if they do mate, they deposit unfertile eggs. Again, the evidence points strongly to disease in the cultures.

Now let us turn to the broods which reveal some measure of success. Taking his families C, D and G separately and differentiating between spring and summer broods, we find that of Hughes' family C, the experimental spring broods, treated as well as control, yielded 2914 ova of which 39·3% gave rise to larvæ and 19·7% to imagines—a death rate of 80·3%. In the summer batches 3755 eggs were laid giving 37·5% larvæ and 6·1% insects, a death rate of 93·9%.

Similarly, the spring lots of family D supplied 2765 eggs from which there originated 44·3% larvæ and 25·7% moths, a death rate of 74·3%,

the comparative figures for the summer lots being 2967 ova, 31·2% larvæ and 9·1% imagines, a death rate of 90·9%. Furthermore, in family G, the spring batches included 4676 eggs followed by 36·4% larvæ and 16·2% insects, with a death rate of 83·8% and the summer broods 6683 eggs with 39·9% larvæ and 5·9% moths, a death rate of 94·1%.

Summing all the broods together, we have a grand total of 23,760 eggs, yielding 38% larvæ and 12% imagines—a loss of 88%.

Or, to put it otherwise, including all broods, treated and control, the insignificant average of 15·1 insects per brood has been reared. One can assert with safety that no other cause than disease suffices to account for losses of such a magnitude.

In my own experiments (1928 and 1934), the treated batches of the first-named series comprised 649 ova from which I reared 389* insects, with a mortality rate of only 40%, whilst, in the second, I secured 898 ova and 696 imagines, with a death rate of 22·5%.

With 28 untreated spring broods (introduced from another series for the sake of comparison only) and the 21 control (summer) broods I encountered death rates of 22% and 29% respectively. Death rates of 40%, 22·5%, 22% and 29% can only serve to emphasize the deductions made from Hughes' figure of 88%.

If it be regarded as proved that Hughes' cultures carried disease unrecognized by him, the bearing of that fact on the success or failure of his experiments comes up next for consideration.

To throw light on this was the aim of my experiments in which disease was introduced into cultures known, under healthy conditions, to be certain to yield melanics. For this purpose three batches of larvæ, resulting from the mating of homozygous melanics, descended from family *d* of the 1928 work, with heterozygous types and three from the mating of heterozygous types *inter se* were taken. These, in ordinary circumstances, ought to give rise to 50% and 25% melanics respectively. Now, it happened that just before this work was planned, a stock of larvæ of *Selenia lunaria*, developed a disease which subsequently turned out to be due to a sporozoan parasite. Affected larvæ of this species were therefore confined with each culture of *S. bilunaria*; without exception, these were infected. However, to give the diseased larvæ every chance, instead of rearing them in cages indoors, I sleeved them outside on hawthorn in huge linen sleeves with results given in Table IV.

As will be seen, two of the batches derived from heterozygous parents produced no melanics, and one a single black specimen, whilst the matings

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* This includes one rather small brood reared under winter conditions when suitable food was difficult to obtain.

of homozygous blacks with heterozygous types ended in 3, 2 and 4 melanics respectively.

The bulk of insects from these cultures was destroyed immediately, but a dozen of the types taken from the broods D, E, and F were mated. From these pairings, 300 mixed larvæ were taken and sleeved outside. Of the insects so reared, 49 were typical and 1 melanic—a very significant result when one considers that, theoretically, a majority of the matings should have been between heterozygous types.

TABLE IV—BROODS INTO WHICH DISEASE WAS INTRODUCED

Family	Type of mating	Number of eggs	Female		Male	
			Type	Melanic	Type	Melanic
A	T*t* × *tt*	184	12	1	21	2
B	T*t* × *tt*	153	9	0	12	2
C	T*t* × *tt*	176	15	1	23	3
D	T*t* × T*t*	167	15	0	20	1
E	T*t* × T*t*	119	11	0	16	0
F	T*t* × T*t*	128	9	0	14	0
Batch G	T*t* × T*t* or TT × T*t*	300 mixed larvæ	20	0	29	1
	Totals		91	2	135	9

* TT = Homozygous type; T*t* = Heterozygous type; *tt* = Homozygous melanic

The import of the facts presented by Table IV is unmistakable. In cultures of larvæ, normally destined to yield certain well recognized proportions of melanics, the effect of the introduction of sporozoan disease has been to exercise a preferential elimination of melanics. If this is the case in broods which yield such high figures as 50% and 25% of melanics, what would be the effect of such a disease on treated experimental batches in which only an odd larva or two can, at the most, be expected to deliver melanic imagines ?

In my opinion, no matter what the disease carried by Hughes' cultures was, *it was potent enough to reduce his broods to negligible proportions and to kill off any potential melanics*; hence the failure of his work.

With only one further remark and my criticism of Hughes' work will finish. That worker appears to be under the impression that the manganese in industrial areas is believed to occur *in* the leaves of various plants; had he read my preliminary paper (1920), he would have noted that it is regarded, nay proved, to form a film on the foliage.

The Work of Thomsen and Lemcke—It is difficult for one to understand how these experiments can be regarded as exact repetitions of the work of Garrett and myself, if due regard be paid to the fact that their first treated generation received hawthorn as food, the second raspberry, the third rose followed by hawthorn, and the rest hawthorn. If, however, this objection be waived, my criticisms would be much the same as for those of Hughes. They report in the successive broods of their controls death rates of 78%, 91%, 83%, 70·5% and 81·3%, respectively, and of their treated lots of 70%, 97·2%, 82·2%, 84·2% and 85·8%, although these huge mortalities are masked by the figures they supply being based on young larvæ and not on ova.

However, in addition to disease, defects in their technique, which some of my students have seen personally, seem to be responsible in part for some of their losses. In the first place, although *Selenia bilunaria* remains very healthy in most breeding cages, it resents being reared in glass jars, for there the resultant excess moisture not only affects the food, but in addition, keeps the air within too moist. The use of paper covers does not obviate the difficulty, for they do not permit the free circulation of the air within; thus sporozoan disease is favoured. A further source of loss lies in the method of wintering the pupæ. *Selenia bilunaria*, like most other species, is injured by removal from the cocoon before the winter; the cocoon, nevertheless, may be discarded with impunity just before the emergence of the moths.

The Experiments of Walther—Neither the technique nor the mortality rates in Walther's cultures are open to criticism, as he supplies no facts concerning them. Moreover, no figures are offered in respect to individual broods, the whole of his results appearing in the form of generation totals.

Walther himself regards his experiments as fruitless; in spite of that fact, he records that, amongst the treated insects which he labels as F_4II, aberrant specimens put in an appearance; these displayed in less striking examples, very broad and dark "middle shades" and, in more extreme cases, the disappearance of the pattern except for these exaggerated "middle shades." The most strongly aberrant moths he describes as " einfarbig ledergelb."

These insects he evidently did not recognize as melanics; nevertheless, as such they are to be regarded, for they appeared freely amongst the melanics recorded by Harrison and Garrett (1926) and Harrison (1928, *a*), rarely in the spring brood but more freely in that bred in July. Similarly, some of the insects discussed by Mansbridge (1927–28) present the same facies.

Most unsatisfactorily, Walther seems not to have segregated these exceedingly interesting examples for breeding work, but contents himself with remarking that the form disappeared in the following summer generation to appear sporadically in the following spring batches. This demonstrates with some degree of certainty that their characteristics had been inherited in a manner similar to a Mendelian recessive.

Such insects, taken from my own cultures, are figured in the last row on fig. 1, Plate 13.

The Criticisms of Sinnott and Dunn (1925), *Muller* (1929) *and Sonneborn* (1930)—Sinnott and Dunn (1925) make the comment "Harrison has fed normal moths (*sic*) with metallic salts in an endeavour to induce melanism, but his experiments do not prove that melanic forms originated in this way." As this remark was made before the appearance of the original paper by Harrison and Garrett, the criticism is open to objection owing to its having been made without knowledge of the full facts of the case, and for this reason no further comment seems necessary.

Muller (1929 and elsewhere) equally dogmatically asserts, "Included amongst the treatments were heavy doses of manganese and of lead salts, which had been claimed by J. W. H. Harrison (on the basis of what appeared to me genetically unconvincing data) to produce visible mutations in butterflies." As no such claim was ever made by me, it appears that Muller has confused my experiments in which the butterfly *Pieris napi* was involved, with those dealing with the moth *Selenia bilunaria*, simply because the two papers were sent out as one separatum. In any case, an *ipse dixit* of this type cannot be regarded as satisfactory criticism; before it was made, pertinent reasons should have been advanced for it. It is best answered by reference to Haldane's (1932) appendix to Hughes' paper, and to Muller's (1932) rejoinder to Heribert-Nilsson's (1931) reasoned criticism of his own induction work on *Drosophila*.

As for Sonneborn's (1930) remarks, they contain definite internal evidence, not only that he was unacquainted with my 1928 experiments, but also that his knowledge of the 1926 work was derived from a short series of comments made by me (1928) on certain experiments of Mansbridge (1927).

Sonneborn very correctly begins by pointing out that Harrison and Garrett claim to have induced melanism by feeding larvæ on food impregnated with lead nitrate. He continues by making the further statement that, because Mansbridge claims to have produced melanic forms by repeated selection, doubt is thus thrown upon the genetic purity of their stocks, and further adds that in *Selenia bilunaria* no
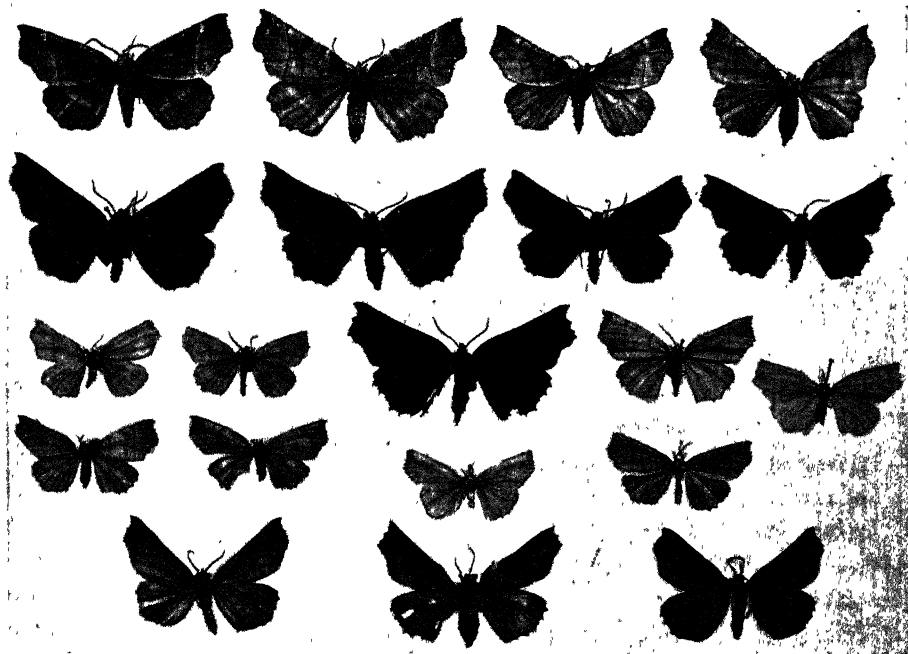
FIG. 1—First row: ♂ and ♀ spring brood *Selenia bilunaria*; ♂ and ♀ summer brood *S. bilunaria*. Second row: ♂ and ♀ spring melanic *S. bilunaria*; ♂ and ♀ summer melanic *S. bilunaria*. Third row: first two from batch S; middle, induced melanic of 1933; fourth from family W; fifth from S. Fourth row: first two from T; middle albino ♂ from T; fourth from V. Fifth row: insects resembling Walther's aberrant forms. Many of the insects have been used for breeding purposes, and some, especially the first two and last one in the third row, are therefore worn. Wing expanse 7/10 natural size.

melanics had been observed in nature. Had Sonneborn really studied the works he ventures to criticize, he would have discovered that, whilst Mansbridge's notes appear on p. 22 of the ' Proceedings of the Entomological Society ' for 1927, on the following page Cockayne (1927–28) gives an account of natural melanic *Selenia bilunaria* originating in W. Yorks.

Next, although he recognizes in his opening statement that " the induced melanism . . . was inherited as a simple Mendelian recessive," he proceeds to urge that the ratios* of types to melanics in the critical batches are explicable on the basis that they are governed by a multiple factor system. As proof he insists that Federley (1920) had discovered that such a system is responsible for the facts noted in *Spilosoma lubricipeda*. This has just as much bearing on the argument as the observation that in *Tephrosia consonaria* melanism is dominant.

In addition, he insists that selective mortality amongst the ova would have rendered such ratios possible. This view would never have been expressed had appeal been made to the original papers; the death rate has never been so great as to interfere with the orderly appearance of simple Mendelian ratios in the broods employed in the inheritance tests. Finally, to supply variety to his " explanations " of our aberrant ratios, he states that I (Harrison, 1923) admitted the difficulty of recognizing melanics in one of my species. No admission of this nature was ever made by me, for my (Harrison, 1923) exact words are " New pairings were made of moths so pale that I described them in my notes as ' nearly ' types." To convert this simple statement into the stronger one of Sonneborn's serves to emphasize the need of care in dealing with forms with which one is not acquainted or in criticizing papers one had never seen.

To anyone used to working with Lepidoptera the moths were melanics and nothing more; that my judgment was correct, my breeding experiments conclusively demonstrated.

From these remarkable arguments he reaches the unwarranted conclusion " Thus, neither the fact that no melanics of one species were ever found in nature, nor the fact that melanics appeared in peculiar ratios excludes the possibility that the stocks used by Harrison and Garrett contained latent genes for melanism." In my view, such a deduction cannot be extracted, in a manner consonant with scientific procedure, from the alleged " facts " of Sonneborn.

The Present Experiments. (*a*) *On Melanism*—In this new work, owing to the circumstance that I was in possession of a strain of melanic moths

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* He, however, very carefully mentions the 26 : 1, 29 : 1 ratios, but just as pointedly ignores the inconvenient 12 : 8 result.

unrelated to the research broods, a much more effective system of controls was in operation to guard against the possibility that the experimental stocks carried latent melanism.

Obviously, if the experimental stocks had been genetically impure at the commencement of the work, one member of the original pair must have been heterozygous; one half, therefore, of that insect's brothers and sisters must have possessed the same genetical composition. Outcrossing six of these to homozygous melanics without the development of melanism in their progeny in itself affords weighty evidence of the genetical purity of the strain in respect to melanism. However, four additional consecutive treated generations were controlled similarly also with negative results, except for the single batch, discussed separately below, which clinches the argument. But in each of these cases, on the hypothesis that the original strain had been contaminated, the brothers and sisters must have included equal numbers of homozygous and heterozygous types; it, therefore, becomes, genetically speaking, practically a certainty that the melanism which ultimately appeared was due to induction and not to Mendelian phenomena.

Moreover, the fact that one of the controls to family M_4 did deliver a single melanic, when due weight is attached to the low death rate, demonstrates that only very few gametes contributed by M_4 carried melanism. This, in turn, presents strong proof that they owe their genetical powers to induction.

It seems, then, that we are justified in our conclusion that melanism has been induced in the strain derived from family A; if that is granted, then the results of the 1926 and 1928 work are upheld and confirmed.

(b) *On Albinism*—In this section of the work, as we have perceived, the effects of parental starvation have manifested themselves not only in the parents but in the offspring likewise. Clearly, this may be regarded as a case of persistent injury to the germ plasm. Now, in discussing recent work on induced mutations in *Drosophila*, there has been no hesitation in regarding the recurrence of the induced characters in the descendants of treated insects as inheritance, although at least some of the cases are admitted as due to germinal damage. Similar treatment cannot be denied to these albinistic moths, and we may reasonably state that the abnormal characters shown in the generations succeeding the treated ones are inherited. That being so, since the first treated insects themselves display the same characters, the circumstances admit of two possible explanations: (1) that based on appeal to Lamarckian principles, and (2) that offered by

" parallel induction." Whilst the former cannot by any means be excluded, my inclinations are toward the second.

Attention should, however, be directed to the slight tendency for the effects to become very slightly less intense in later generations. The experiment, of course, falls in line with those of Kellogg (1908).

I have to thank the Research Committee of Armstrong College for a grant in aid of these and other researches.

5—SUMMARY

A further induction of melanism in the Geometrid moth *Selenia bilunaria* esp. is described.

Experiments in starving wild larvæ of the same insects have resulted in the production of exceedingly small moths with the sexual and seasonal dimorphism suppressed.

These characteristics persisted, with but slight amelioration, for several generations, even when the larvæ were fed on the most nutritious of foods.

It has been shown that the introduction of sporozoan disease into healthy cultures of larvæ, known from the genetical constitution of the parents to be capable of yielding melanics and types in certain definite ratios, has the effect of bringing about the differential elimination of melanics.

The failure of Hughes, and Thomsen and Lemcke to induce melanism is assigned to the very high death rates displayed by the whole of their cultures, these being probably due to disease, coupled with, or depending upon, defects in technique.

It is pointed out that Walther has succeeded in inducing melanism in the same species.

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The Integration of Plant Behaviour

V—Growth Substance and Traumatic Curvature of the Root

By Sir FREDERICK KEEBLE, C.B.E., Sc.D., F.R.S., and M. G. NELSON, M.A.

1—INTRODUCTION

The manner of response of the root to injury is well known. A lateral wound made within 1 mm or 2 mm of the extreme tip gives rise to a negative curvature: the root curves away from the wound. .A similar injury made elsewhere in the growing region evokes a positive curvature; the root curves towards the wound. The curvatures are manifestly growth curvatures. Like those induced by gravity, traumatic curvatures are the consequence of unequal growth of opposite sides of the region of elongation. In the one case they are undoubtedly brought about by the stimulus of gravity; and in the other they are supposed also to owe their origin to a stimulus, a wound stimulus. But, whereas something at all events is known of the way in which the stimulus of gravity acts on the root, the mode of operation of the wound stimulus—if stimulus there be— remains obscure.

The Went-Cholodny hypothesis of geotropism which is supported by Cholodny's experiments (1924, 1926), those of the authors in collaboration with R. Snow (1931), of Boysen-Jensen (1933, *a, b*), and others (Snow, 1932), attributes to growth substance contained in the root an essential part

in geotropic curvature. It holds that growth substance which inhibits the growth of the root is a normal secretion of- the root tip. Produced continuously by the tip, it passes upwards by straight paths and reaches all parts of the region of elongation. Although in the passage through the elongating region the concentration falls off progressively, the distribution of growth substance at any given level is uniform and therefore, the inhibitory effect being equal on all sides, the unstimulated root continues to follow a straight downward path. When, however, the root is exposed to the stimulus of gravity the uniformity of distribution of growth substance is disturbed; more is found to occur on the lower than on the upper side of the tip and the inequality of distribution is held to be due to a passage downward from the one side to the other. Since the lower side of the tip now contains more, and since growth substance travels from tip to elongating region by straight paths, the lower side of the elongating region comes also to contain more than the upper side; the upper side grows faster than the lower and the root curves downward.

How the stimulus of gravity operates in bringing about the unequal distribution of growth substance in the upper and lower side of a horizontally placed root tip is uncertain; but whatever the mode of operation may be it is evident that the inequality on the two sides is a result of the excitation set up by the stimulus.

The part played by growth substance in geotropic curvature led the authors to enquire whether that substance may not be found to play a decisive part also in the traumatic curvature of the root; as indeed it has already been shown to do by Weimann (1929) in the traumatic curvatures of the coleoptile of the oat.

The occurrence of growth substance in the root of *Zea Mais* has, however, been denied by Gorter (1932) and therefore further evidence that growth substance does occur in the root of this plant is given (Section 2 (A)). In that section also evidence is produced (2 (B)) to show that the growth inhibitory substance previously described by Keeble, Nelson and Snow (1930) as occurring on the surface of amputated root tips is an exudation of preformed growth substance and not, as originally suggested by them, a wound hormone secreted by the injured tissues.

Section 3 describes experiments on the localization of the receptor of the stimulus of gravity.

Section 4 gives an account of experiments which lead to the conclusion that the distribution of growth substance and the effect that wounding has upon it suffice, without the invocation of a specific wound stimulus to account for the traumatic behaviour of the root.

2—GROWTH SUBSTANCE

(A) *Occurrence in* Zea Mais

The experiments from which Gorter concluded that the root tip does not produce growth substance were made by a method which gives positive results when applied to the coleoptile. The method consists in standing a coleoptile tip on an agar block which absorbs the growth substance contained in it. The block is then placed excentrically on a freshly amputated coleoptile which in turn absorbs the growth substance and indicates by its appropriate curvature that it has done so. Repetition of the experiment with root tips of *Zea Mais* gave in Gorter's hands negative results.

Cholodny (1933) has already advanced what appear to be good and sufficient reasons why Gorter's conclusions cannot be accepted. In as much, however, as Boysen-Jensen (1933, *a*) has concluded that although the root tips of *Vicia Faba* contain a fair amount of growth substance, those of *Zea Mais* in their natural state may contain little or none, it became necessary to make a further investigation of the root of *Zea Mais*.

The method employed by us was to cut out rectangular blocks of 20% gelatine about 0·5 mm thick and large enough to allow twenty or more tips to stand upon them. The tips were taken from roots of *Zea Mais* seedlings decapitated at 1·5 mm from the extreme tip. They were placed upright with their cut surfaces on the gelatine blocks in numbers sufficient to cover the blocks. The blocks were kept for 1 hour in a damp atmosphere at 20° C. They were then cut up into as many pieces as there had been tips standing on them, and each piece was placed excentrically on the cut surface of the root of another maize seedling decapitated at 1·5 mm from the tip. As controls, the roots of similar seedlings were similarly decapitated and blocks of 20% gelatine on which no root tips had stood were placed on their cut surfaces in a similar manner. The seedlings were arranged with their roots vertical in a damp atmosphere in a large glass vessel kept at 20° C.

After 24 hours it was found that amongst the roots of the first series, 29 out of 33 had curved towards the side covered by the gelatine, and none had curved in any other direction. The mean curvature amongst the 29 roots that had curved was 43·2°, and the extremes were 16° and 90°. Twenty of the roots had already curved in the first 6 hours. On the other hand, amongst the controls, after 24 hours 28 out of 31 roots showed no curvature though the remaining 3 roots curved towards the

side covered by the block, the mean curvature being 43·3°. It is uncertain how the three curvatures should be explained, but it is probable that they were due to some impurity in the gelatine.

These results, like those previously obtained by Hawker (1932), show that the root tips secrete into gelatine a substance which, when the blocks are applied excentrically to the cut surfaces of decapitated roots, causes the roots to curve towards the side covered by the blocks. The simplest interpretation of this fact is that the root tip secretes growth substance which travels up the decapitated roots on the side to which it is applied and retards the elongation of that side.

We also performed experiments that were similar except that coleoptile tips of maize were placed upon the gelatine blocks instead of root tips. The gelatine blocks on which the coleoptile tips had stood often caused the root stumps to curve towards the side covered by them, though the curvatures were less numerous than when root tips were used.

From the former experiments it is to be concluded that the root tip of *Zea Mais* contains growth substance which inhibits growth of the root.

(B)   *The Origin of " Wound Substance "*

The origin of the growth inhibitory substance (wound substance) which occurs on the cut surface of an amputated root was investigated in the two following ways.

(1) *Comparison of the Rates of Growth of Intact and Amputated Roots—* The relative rates of growth of intact and amputated roots should provide evidence of the origin of the " wound substance." For if it is an exudation of growth substance which already existed in the intact root tip, amputation, by cutting off supplies, should result in smaller amounts reaching the elongating region and the amputated root should therefore grow faster than the intact root; but if on the other hand the wound substance is a special secretion from the wounded tissues, then either some of it will reach the elongating region and the root will grow more slowly, or none will reach that region and the growth of the amputated root will be equal to that of the intact root, unless indeed the inhibitory effect of operative shock intervenes, in which case amputation will again result in a slowing down of growth.

Previous observations on the operative shock which occurs after amputation had shown that the root of *Zea Mais*, in which about 75% of the total growth is concentrated in 2 mm zones (the 2nd and 3rd), is unsuitable for the experiment. The root of *Vicia Faba*, with a more diffuse elongating region extending over some 6 mm, is less affected by

operative shock and accordingly the comparative growth measurements were made on this plant. The roots used were those of seedlings of *Vicia Faba* which had germinated in sawdust under constant conditions. They were picked out in pairs. The roots of each pair were approximately of the same length (about 1 inch), and were growing at the same rate. One of each pair was so amputated as to remove as exactly as possible 1 mm of the apex. Each root was marked with Brunswick Black at intervals of 1 mm in order that the distribution of growth in the successive zones as well as the total growth could be measured. The roots were grown in a thermostatically controlled dark chamber and measured at intervals.

The results recorded in Table I show that the amputated roots grow faster than the intact roots. The average rates expressed in percentages are: intact 100, amputated 119·86% or, deducting the amount of growth of zone 1 which zone was removed from the amputated roots, the relative percentages are: intact 100, amputated 120·2. It is to be noted also that of the 10 pairs, intact and amputated, six show growth in favour of the amputated roots, two equality, and two show in one pair a slight, and in the other a distinct superiority of growth of the intact roots. The distribution of growth in the successive zones is also noteworthy. In all the millimetre zones, save the second, that in which the inhibitory effect of shock is most pronounced, the average growth of the amputated root exceeds that of the intact root. Furthermore, the amputation leads occasionally to a slight resumption of growth in the distal part of the elongating region—the 7th zone—where in the intact root growth has ceased; thus indicating that growth substance plays a part in co-ordinating growth in the different parts of the elongating region.

The results of the experiment lead to the conclusion that the growth substance which appears on the surface of a wound made anywhere in the growing region is, at all events mainly, an exudation of the growth substance preformed in the root and is not, at least in the first instance, a product of the metabolic activity of the wounded tissues.

(2) *Semi-amputation*—The results of cutting half-way through the root tip by a section at right angles to the long axis should also give decisive evidence for or against the exudation hypothesis. For if the root tip contains no growth substance the result will be either that the semi-amputated root curves towards the cut if wound substance reaches the elongating region, or the root will not curve at all if none of the wound substance reaches the region of elongation. But on the other hand if growth substance occurs in the intact root the loss of some of it by

exudation will result in more rapid growth of the cut side and that side will therefore curve away from the cut.

Our first experiments gave conflicting results because, as was found subsequently, cutting does not stop entirely the upward passage of growth substance on the cut side. When, however, the upward move-

TABLE I—THE RELATIVE GROWTH OF INTACT AND AMPUTATED
ROOTS OF *Vicia Faba*

Growth in 24 hours

	1st zone	2nd zone	3rd zone	4th zone	5th zone	6th zone	7th zone	Total gain
				Intact Roots				
1	—	0·25	0·5	1·0	3·0	0·75	—	5·5
2	—	0·25	2	5	2	0·25	—	9·5
3	—	0·25	2·25	2	0·25	—	—	4·75
4	—	1	2·25	1	0·25	—	—	4·5
5	—	0·25	2·25	3·0	2	0·5	—	8
6	—	0·75	2	2	1·25	0·5	—	6·5
7	—	0·75	2	3	0·75	—	—	6·5
8	—	1·75	3·75	1	0·75	0·5	—	7·75
9	0·25	1·75	3·75	3	1	0·75	—	10·5
10	—	1·5	3·25	3·25	1	0·5	—	9·5
Average	0·025	0·85	2·4	2·425	1·225	0·375	—	7·30 (100%)
				Amputated Roots				
1	·	1·5	2·75	1·5	0·5	—	—	6·25
2	—	0·5	2·75	2·75	2	1·0	0·5	9·5
3	—	0·25	3·75	2	1	0·25	—	7·25
4	—	0·25	4·5	2·75	2·25	1	0·75	11·5
5	—	0·75	4·5	2·5	1	0·5	—	9·25
6	—	—	2	2	1·5	0·75	0·25	6·5
7	—	—	3·5	3	2	1	—	9·5
8	—	0·5	3	2·5	1	0·25	—	7·25
9	—	—	3	6	2·5	1	—	12·5
10	—	1·5	5	1	0·5	—	—	8
Average	—	0·525	3·475	2·60	1·425	0·575	0·150	8·75 (119·86%)
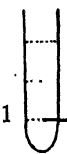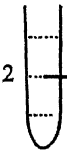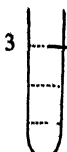
ment was prevented by a mica slip inserted between the cut surfaces, the roots which were cut half-way through at 1 mm from the extreme tip gave consistently definite results.

Semi-amputation at 1 mm. (*a*) *Vicia Faba*, Table II—Ten roots were cut half-way through at 1 mm from the extreme tip and fixed in a moist

chamber in the vertical position. After 6 hours five roots, and after 24 hours all 10 showed negative curvature.

(b) *Zea Mais*, Table III—Thirty-nine roots were treated in a similar manner. In 6 hours 12 showed negative curvature and 27 exhibited no curvature. In 2 hours 22 (56·4%) curved negatively, six remained vertical: eight showed positive tip curvature, one a negative tip curvature and two S-shaped curvature.

TABLE II—SEMI-AMPUTATION OF ROOT TIPS, *Vicia Faba*

	No. of roots	Curvatures in 6 hours		Curvatures in 24 hours			
		Stump	Tip	Stump		Tip	
		Negative	Positive	Negative	Positive	Negative	Positive
1	4	2	0	4	0	0	0
	6	3	0	6	0	0	0
	10	5		10			
2	4	3	0	4	0	0	0
	6	3	0	3	1	2*	0
	10	6		7	1	2*	
3	4	0	1	0	0	0	3
	6	0	1	0	0	4 1(S)	0
	10		2			4 1(S)	3

* Showed swellings above mica slip.
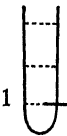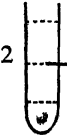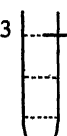
The results with *Zea Mais* and *Vicia Faba* demonstrate that roots cut half-way through at 1 mm from the apex and placed vertically, if they curve at all in the first 6 hours curve negatively, away from the cut. In the case of *Vicia Faba* all the roots showed negative curvature within 24 hours; but with *Zea Mais* some showed either S-shaped curvatures or a positive or negative tip curvature.

The behaviour of roots in which the semi-section is made at greater distance from the apex is described on p. 118, where reasons are given

for associating the tip- and S-shaped curvatures just described with the change in position of the elongating region with respect to the place of semi-section which growth brings about automatically and progressively in the course of the experiment.

If this explanation is accepted it is to be concluded that semi-section at 1 mm from the apex induces negative curvature in vertically placed roots:

TABLE III—SEMI-AMPUTATION OF ROOT TIPS. *Zea Mais*

No. of roots	Curvatures in 6 hours		Curvatures in 24 hours				
	Stump	Tip	Stump			Tip	
	Negative	Positive	Negative	Positive	Others	Negative	Positive
6	4	0	5	0	0	0	0
6	2	0	2	0	0	0	4
7	2	0	4	0	0	1	1
8	1	0	6	0	0	0	1
12	3	0	5	0	2*	0	2
39	12		22		2*	1	8
6	0	2	0	0	0	0	6
6	0	0	0	0	1	0	3
7	0	1	1	0	1	0	3
8	0	2	0	0	0	0	8
10	1	4	1	1	0	2	6
37	1	9	2	1	2	2	26
6	0	4	0	0	0	0	6
7	0	2	0	0	0	3	4
8	0	1	0	0	0	2	6
12	0	3	0	0	0	2	7
33		10				7	23

* S-shaped curvatures.

a result in accordance with expectation on the hypothesis that wound substance is an exudation of growth substance preformed in the intact root.

3—THE RECEPTOR REGION OF THE STIMULUS OF GRAVITY

A root amputated by a transverse cut made 1 mm from the extreme tip rarely if ever shows geotropic curvature even after it has been in a hori-

zontal position for 24 hours (Snow, 1923). It might therefore be concluded that the amputated root is insensitive to the stimulus of gravity and that the insensitiveness is due to the removal by the amputation of the receptor of the stimulus. This conclusion, however, cannot be accepted without further investigation. For, on the Went-Cholodny growth substance hypothesis, the failure of the amputated root to curve when exposed to the stimulus of gravity might be due to one or other or both of two causes, namely, the absence of a receptor and the lack of sufficient growth substance for the establishment between the upper and lower sides of the stump of a gradient of concentration steep enough to bring about curvature.

Experiments already described (Keeble, Nelson and Snow, 1929) ndicate that the root amputated at 1 mm from the extreme tip still serves as a receptor of the stimulus of gravity and that its failure to curve whilst horizontal is due to insufficiency of growth substance. The experiments show that an amputated root which had failed to curve whilst in a horizontal position, when reheaded with a coleoptile tip and placed vertically curves toward the side which was lowermost when the root was horizontal; that is it makes a belated geotropic curvature.

It would seem, therefore, that the stimulus of gravity does set up in the amputated stump an excitation similar to that which it evokes in the intact root; but that the excitation remains ineffective until the stump has received enough growth substance from the coleoptile tip for the previous excitation to take effect in geotroptic curvature.

These conclusions are confirmed by the results of experiments now to be described.

Split Tip Experiments

The extent of the receptor region was investigated by observing the curvatures of roots split in a median plane to a known depth from the extreme tip and then exposed to the stimulus of gravity. A mica slip inserted in the cut ensured that no growth substance passed from one side of the split tip to the other and therefore if normal geotropic curvature takes place it must be ascribed, in default of other explanation, to excitation and a consequent redistribution of growth substance in the intact part of the root. It is well known, however, that the split tips of roots may show abnormal curvatures; for example, the split parts may curve independently of the intact parts and not infrequently bend away from one another. It was therefore necessary to ascertain whether these abnormalities occur so frequently and so quickly as to make the method untrustworthy.

The results of the following experiments show that this is not so.

Twenty-six roots of *Vicia Faba* were split to a depth of 1 mm from the extreme tip. After 6 hours 23 showed no abnormal curvature of the tips and 3 (11·5%) showed a gaping apart of the two sides of the split tip. After 24 hours, 16 showed no abnormal curvature and 10 (38·4%) showed gaping.

When the split extended to a depth of 2 mm the results with 30 roots were: after 6 hours, normal 25; gaping tips 5 (16·6%), though after 24 hours 24 out of the 30 roots exhibited abnormal curvatures of the tip.

Even with roots split to a depth of 3 mm the abnormal curvatures were relatively few in the course of the first 6 hours. Thus out of 45 roots 39 were normal and 6 (13·3%) showed a gaping of the tip; whereas after 24 hours only 9 root tips had remained normal, the remainder (33) showing abnormal curvature of the split tips.

But even the roots with gaping tips may show—the tip apart—a definite harmonious curvature involving a good many millimetres of the intact part of the root, for as the experiment proceeds more intact tissue becomes involved in growth.

It is therefore concluded that provided reliance is placed only on strictly harmonious curvatures comparable with those of intact roots the method is trustworthy.

In the experiments now to be described the split roots were placed in a moist chamber at constant temperature with the plane of the cut horizontal.

TABLE IV

Time	No. of down curves	%	Average down curvature	No. of horizontal roots	No. of up curves
hrs			°		
6	24	44·4	23·8	29	1
24	47	87·0	35·9	5	2

Experiment 1. *Zea Mais*, 1 mm split—Fifty-four roots were split to a depth of 1 mm from the extreme tip. The split passed through root cap, vegetative apex (formative zone) which together occupy the first 0·5 mm of the tip, and extended a little way into the region of elongation which begins about 0·5 mm from the tip. The roots placed horizontally, showed the results given in Table IV.

Experiment 2. *Zea Mais*, 1 mm split—In a second experiment 35 pairs of roots were used, those of each pair being of equal length and rate of growth. One of a pair was split to a depth of 1 mm, the other

remained intact. Each pair was placed simultaneously in a horizontal position in the dark chamber. The results are given in Table V.

TABLE V

Time	No. of down curves	%	Average down curvature	No. of horizontal roots	No. of up curves
			35 Split Roots		
hrs			°		
2	5	14·3	12·2	30	—
4	10	28·6	17·4	25	—
6	15	42·8	22·5	17	3
24	31	88·5	32·4	1	3
			35 Intact Roots		
2	16	45·7	18·7	—	—
4	33	94·3	35·8	—	—
6	35	100	47·1	—	—
24	35	100	56·8	—	—

The percentages of split roots that curved downward and the average curvatures are very much the same in the two experiments both at 6 and at 24 hours. The results obtained in experiments 1 and 2 after 6 hours are given in Table VI.

TABLE VI

	No. of down curves	Average curvature
	%	°
Experiment 1. Split roots	44·4	23·8
Experiment 2. Split roots	42·8	22·5
Experiment 2. Intact roots	100	47·1

That is to say the response to the stimulus of gravity after 6 hours is about half as great in the roots with root tips split 1 mm as it is with intact roots.

Experiment 3. *Vicia Faba*, curvatures of 1 mm split roots and intact roots (12 pairs), horizontal.

The figures in Table VII show that as with *Zea Mais* the numbers of split roots that curve in 6 hours and the average amounts of curvature are about half those of the intact roots. It seems fair to use the figures at 6 hours since in the earlier period the operative shock-effect is likely to disturb the results, and also because prolonged stimulation of intact

roots, as is well known, may be accompanied by a decrease in the rate of curvature.

<p align="center">TABLE VII</p>

Time, hours	Number of curved roots		Average curvature	
	Split %	Intact %	Split °	Intact °
2	8·3	41·6	14·0	12·8
4	25·0	91·6	16	38·6
6	50	91·6	19	43·5
24	91·6	100	26·3	43·3

Thus in *Zea Mais* and *Vicia Faba* roots split to a depth of 1 mm curve geotropically. The rate of curvature is at first very slow, but after 6 hours becomes about half that of intact roots. The splitting, although it may be without effect on the receptor of the split tip, manifestly prevents the result of excitation of it—the redistribution of growth substance between the upper and lower sides of the split part—from taking place. If, therefore, a downward migration of growth substance is the cause of the inequality of concentration between the upper and lower sides of the root, the migration in the split roots must take place in the still intact part of the root. The experiments show that this—the intact part of the 1 mm split root—brings about geotropic curvatures about half as large as those made by the intact root. Therefore the capacity for excitation of the intact part of the split root must be about equal to that of the more apical part put out of action by the splitting. If this be so, it follows that receptivity of the stimulus of gravity is diffused uniformly from the tip to a distance of more than 1 mm, which region not only includes the vegetative apex with its root cap but also at least a part of the region of elongation.

On this view, if the statocytes of the root cap are to be regarded as playing a part in geotropism, they must act by affecting the rate of secretion of growth substance and not on the receptor.

Experiment 4. *Vicia Faba*, 2 mm split—Experiments in all respects similar to experiment 2 were made by splitting roots to a depth of 2 mm.

Roots split to this depth still showed themselves capable of bringing about harmonious geotropic curvature though occasionally up curvatures were produced. Thus 16 pairs of split and intact roots gave the following curvatures:—

After 6 hours—16 split roots: 10 downward curves, average 17·7°.
             1 upward.
             16 intact roots: 16 downward curves, average 47·1°.

After 24 hours—16 split roots: 13 downward curves, average 33·5°.
1 upward.
16 intact roots: 16 downward curves, average 46·8°. ·

Inability to secure fresh seed—without which no trustworthy results may be obtained—prevented more than one small experiment being made with *Zea Mais*. The result, nevertheless, is given.

Experiment 5.   Zea Mais, 2 mm split—
After 6 hours—5 split roots: 2 downward curves, average 22·5°.   1 upward.
5 intact roots: 5 downward curves, average 55·6°.
After 24 hours—5 split roots: 3 downward curves, average 51·6°.   2 upward.
5 intact roots: 5 downward curves, average 49·8°.

So far as the results both with *Vicia Faba* and *Zea Mais* show numerous and large downward curvatures they indicate that the receptor region extends from the tip well into the region of elongation.

Experiment 6.   Vicia Faba, 3 mm split—Roots split in a median longitudinal plane to a depth of 3 mm also give harmonious downward curvatures.   During the first 6 hours of an experiment relatively few of the roots show gaping of the tip which becomes very pronounced and general after 24 hours, but when it does occur the gaping does not involve the whole of the split part.   The consequence is that the split tip shows in its more distal part a downward curvature, and the more apical part a gaping of the two halves.   In roots split to a depth of 3 mm the downward curvature is mainly, although not entirely, confined to the split part.   Accepting the current belief that excitation by the stimulus of gravity gives rise to a downward migration of growth substance, it is to be concluded as a result of the split tip experiments that the receptor region extends deeply into the region of elongation and may be co-extensive with it.   If this prove to be so, receptivity of the stimulus of gravity may be regarded as a property of all tissues capable of growth; a conclusion which receives some support from the well established fact that plant members in which growth has ceased may be stimulated by gravity to resume growth and respond by curvature to the stimulus.

4—TRAUMATIC CURVATURE AND GROWTH SUBSTANCE

(A)   *Negative Curvature*

(*a*) *The Region of Response*—The method used for delimiting the region of the root which responds to wounding by negative curvature

and for comparing the intensity of response in different parts of that region consisted in amputating obliquely series of similar roots at varying distances from the apex, and comparing the curvatures made by the roots of a series.

It is essential, however, that the amputations should all be made as nearly as possible at the same angle with the long axis, for, as indicated subsequently, p. 114, small differences in the angle at which roots are amputated make large differences in the amounts of the traumatic curvature they exhibit.

Oblique Amputation—*Zea Mais* seedlings grown in sawdust were selected with roots of the same length and rate of growth. The roots were amputated at 1, 2, 3 or 4 mm from the extreme tip and in all the angle of amputation was as nearly as possible the same: 45°. After amputation the roots were placed vertically in a moist chamber kept at constant temperature.

The results are summarized in Table VIII which also gives for purposes of comparison the average percentage growth in successive millimetre zones of intact roots.

TABLE VIII—OBLIQUE AMPUTATION OF ROOTS OF *Zea Mais*

Region of amputation	No. of roots	5 hours		Next 17 hours		Zonal growth of intact roots
		Growth	Curvature	Further growth	Curvature	
mm zone		cm	°	cm	°	%
1st	8	2·15	383	7·85	473	3
2nd	8	1·45	180	8·30	344	39
3rd	8	0·65	90	1·45	131	44
4th	8	0·4	0	0·3	0	12
5th						2

No curvatures other than negative curvatures occurred, and the curvatures were confined to the roots amputated in the 1st, 2nd and 3rd mm zones. That is to say the power of response to wounding extends for some 3 mm from the extreme tip. This region includes the root cap and vegetative apex, which together occupy 0·5 mm from the extreme tip and the part (2nd and 3rd mm zones) in which elongation is most active. It does not, however, include the whole of the elongating region, for as shown in Table VIII, elongation amounting to 12% of the total takes

place in the 4th mm zone of intact roots, and in amputated roots this zone is responsible for more than 12% of the total growth.

The absence of curvature from the roots amputated obliquely in the 4th mm zone can hardly be attributed to insufficiency of growth of the residual stump.   For roots amputated in that zone made a total growth of 7 mm, which is about equal to that (6·5 mm) of the roots amputated in the 3rd mm zone during the time (5 hours) they were making a total curvature of 90°.   It will be demonstrated presently (p. 118) that roots growing yet more slowly often exhibit well-marked traumatic curvature.

It is therefore more probable that the absence of curvature from the 4 mm amputated stump is due to the smallness of the gradient of concentration of growth substance between the opposite sides, one of which contains more apical tissue than the other.

(b) *Regional Intensity*—Table VIII also shows that the negative curvatures induced by the oblique amputations at 45° decrease with increasing distance from the apex.   The total curvatures made by eight roots in 5 hours were:—

<div align="right">o</div>

Amputation in 1st mm zone 383

,,   2nd   ,, ... 180

,,   3rd   ,, ... 90

That is roughly speaking in the ratio of 4 : 2 : 1.   At the end of 22 hours the corresponding figures, 473°, 344°, 131° show a similar diminution of curvature.   The progressive decrease of curvature may be taken in default of other explanation to be the consequence of the progressive fall in the gradient of concentration of growth substance from the extreme tip to the distal limit of the region of response.

It will be seen, however, that the amounts of curvature and of growth made by the roots during the first 5 hours run parallel with one another. Thus, when the figures given in Table VIII are expressed as percentages, the hourly growth and total curvatures are:—

Amputation	% hourly growth	% curvature
1st mm zone	100	100
2nd mm zone	67·4	46·9
3rd mm zone	30·2	23·4

It might therefore be supposed that amount of curvature is dependent on rate of growth, and hence it will be necessary to ascertain whether rate of growth does actually exercise an influence on curvature or whether the relation is only coincidental, arising from the fact that the successive

amputations, removing as they do increasing amounts of elongating tissue, must of necessity bring about a progressive decline in the rate of growth.

That the parallelism is coincidental is shown by the experiments described in Section 4 (A) (*d*), pp. 113–116, and therefore it may be regarded as probable that the decrease of traumatic curvature is due to the progressive fall of the gradient of concentration of growth substance in the successive parts of the region of response.

(*c*) *Distribution of Growth Substance*—The results of the experiments in oblique amputation admit of simple explanation in terms of growth substance and suggest that not only does growth substance decrease progressively from the extreme tip but also that the decrease is greatest in the 1st mm zone, less but still considerable in the 2nd, and still less in the 3rd mm zone. The fact that the results of other experiments, some of which have been already described, are likewise explicible lends support to the hypothesis. Thus it has been shown (p. 100) that the non-curvature of a root amputated transversely at 1 mm from the extreme tip and placed horizontally may be explained in terms of insufficiency of the growth substance in the stump.

That the concentration of growth substance does decrease from the apex backward had been demonstrated by Boysen-Jensen (1933, *b*). Using other methods he concludes that in *Zea carragua* the relative concentrations in successive segments of the root are:—

1st 2 mm segment	....	98
2nd „	....	78
3rd „	....	34
4th „	....	2

But although the results indicate that concentration decreases, the rate of decrease in the successive segments is too slow to account for the results of our experiments on the relative curvatures of roots amputated at different distances from the apex.

It is to be pointed out, however, that Boysen-Jensen's experiments made on segments 2 mm long give no information of any fall of concentration that may take place within the first 2 mm of the root tip.

The following experiments suggest that the fall in this region will be found to be very rapid.

(1) *Washing Amputated Stumps*—It has been shown in an earlier communication (Keeble, Nelson and Snow, 1930) that the growth of a trans-

versely amputated root becomes faster when the wounded surface is washed with water. Of two similar and similarly amputated roots that from which the exuded growth substance has been washed away grows faster.

The results of our earlier experiments and others made subsequently are given in Table IX.

TABLE IX

Place of amputation	Rate of growth	
	Washed	Unwashed
0·5 mm from extreme tip..............	105·8	100
1 mm from extreme tip	131·5	100
2 mm from extreme tip	169	100
3 mm from extreme tip	141	100

The experiments show that the effect of washing is least in roots amputated at 0·5 mm, and increases progressively in those amputated at 1 and 2 mm.

These results are consistent with the hypothesis that the secretion of growth substance is most active in the most apical tissues of the root, and that the activity of secretion falls off rapidly and progressively in the first 2 mm zones. The root amputated at 0·5 mm, retaining a part of its secretory tissue, continues to secrete growth substance actively after washing has ceased. The secretion makes good the loss due to washing, with the result that the washed and unwashed stumps contain nearly similar amounts of growth substance and accordingly grow nearly at the same rate.

When, however, the root is amputated at 1 mm the larger, and in all probability the most active part of the secretory tissues is removed. The growth substance contained in the stump is mainly that which reached it whilst still in organic connection with the tip. Any loss can be but partly repaired and therefore washing brings about a large disparity between the growth of the washed and unwashed stumps. And still greater is the disparity when amputation is made at 2 mm. With yet more enfeebled powers of secretion—if any powers remain—washing will produce a yet more marked effect. Whether the smaller difference between washed and unwashed roots amputated in the 3rd mm zone as compared with that between the roots amputated in the 2nd mm zone is significant or not cannot be stated. Roots amputated 3 mm from the apex retain but little growing tissue and consequently the amount of growth they make is small, and the effect, therefore, of chance variations will be the more considerable.

Little, if anything, is yet known of the behaviour of growing tissues with respect to growth substance. It may prove that when much is present in tissues which are growing actively, as in zones 2 and 3 of the root of *Zea Mais*, injury leads to a large excretion of growth substance, but that in tissues in which growth is waning the small amount of growth substance that remains in them is held more firmly—as it were in the form of a reserve.

In any case the relative behaviours of the roots amputated at 0·5, 1, and 2 mm are consistent with the hypothesis that the most active secretion of growth substance occurs in the apical part of the 1st mm zone, that it falls off rapidly in the distal part of that zone and ceases altogether somewhere in the 2nd mm zone or just beyond it.

(2) *Traumatic* versus *Geotropic Curvature*—Further indirect evidence is provided by experiments comparing the rates and amounts of traumatic and geotropic curvature made by roots which previous to the experiment were of the same length and rate of growth. According to the growth substance hypothesis the gradient of concentration of growth substance is the mainspring of geotropic curvature. It should therefore be possible by oblique amputation of the tip to provide a gradient as steep as that i nduced by gravity, and by so doing bring about traumatic curvatures at least as rapid and as great as geotropic curvatures.
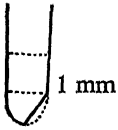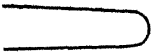
Experiment 1. *Zea Mais*—Twenty-three pairs of similar roots were used. One of each pair was amputated obliquely in the first millimetre zone by a cut which began at 1 mm from the extreme tip and reached the tip in the median line (see diagram with Table X). The effect of the amputation was to remove only a small part of one side of the first millimetre zone. The members of each pair were put at the same time in a moist chamber, the amputated roots being kept vertical and the intact roots horizontal.

As Table X shows, 5 of the 23 amputated roots showed negative traumatic curvature after 2 hours, and a like number of intact roots showed geotropic curvature. The average curvatures were: traumatic, 18·2°; geotropic, 15·6°.
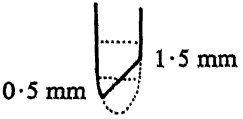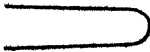
Experiment 2. *Zea Mais*—Other roots comparable with one another but not comparable with those used in the first experiment were arranged in pairs and treated in all respects similarly except that the amputations began at 1·5 mm from the extreme tip and following the same angle of approximately 45° reached the opposite side at about 0·5 mm from the tip. The section removed a large part of the 1st mm zone (see diagram

with Table XI). Although much of the region of active elongation remained the traumatic curvatures were inferior in number and extent to the geotropic curvatures. Thus whereas 6 out of 37 roots curved trau-

TABLE X—COMPARISON OF TRAUMATIC WITH GEOTROPIC CURVATURES.
Zea Mais

No. of roots	No. of negative traumatic curves in 2 hours	Total curvature in 2 hours	No. of roots	No. of negative traumatic curves in 2 hours	Total curvature in 2 hours
	TRAUMATIC			GEOTROPIC	
		°			°
6	1	20	6	1	12
10	4	71	10	2	34
7	0	0	7	2	32
Total...... 23	5	91	23	5	78
Average ..		18·2			15·6

TABLE—XI

	TRAUMATIC			GEOTROPIC	
		°			°
12	4	82	12	10	311
10	1	25	10	3	62
5	0	0	5	2	36
10	1	18	10	3	68
Total...... 37	6	125	37	18	477
Average ..		20·8			26·5

matically in 2 hours, 18 curved geotropically, the average curvatures of the roots that did curve being: traumatic 20·8°; geotropic 25·5°.

In the former experiment all the secretory tissue remained intact on one side of the amputated root; in the latter, part of it was removed and the results are in accordance with expectation if the intensity of secretion

is greatest near the extreme tip and falls off rapidly within the first milli-metre zone.

In as much as the hypothesis serves to explain the results of these experi-ments as well as those previously described, it seems likely that it is true. If so it follows that the negative traumatic curvature of the root is not to be regarded as a response to a specific stimulus but as a necessary conse-quence of the mode of distribution of growth substance in the intact root and of the disturbance of the symmetry of distribution brought about by an asymmetrical wound.

(3) *Oblique Amputation and Reheading.* (α) *With Root Tip*—The results obtained by replacing the obliquely amputated tip on the stump from which it had been severed also show that no specific stimulus is concerned in traumatic response.

Experiment 1. Zea Mais, Table XII—Thirteen roots amputated at 1 mm from the extreme tip at an angle of 45° were reheaded each with its own tip and placed in a vertical position in a moist chamber at constant temperature. Of the 13 roots none showed curvature of the stump after 6 hours; after 24 hours four stumps had curved, two negatively and two positively. When the amputation was done at 1·5 mm from the extreme tip, of nine amputated reheaded roots, none showed any stump curvature in 6 hours and only two in 24 hours; while of the four roots obliquely amputated at 2 mm and then reheaded, none showed any stump curvature whatever.

The replacement of the tip permits of some passage of growth substance from the tip to the region of elongation and with the resumption of the distribution of growth substance to the elongating region the disparity of supplies to the opposite sides brought about by amputation tends to cease, and, consequently, any save occasional and inconsistent curvatures cease also.
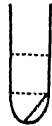
Although the reheaded roots give occasional stump curvature, curva-ture of the reheaded tip frequently takes place. Tip-curvatures are considered on p. 117 where positive traumatic curvature is discussed.

(β) *With Coleoptile Tip*—Experiments were also made with coleoptile tips instead of root tips; the coleoptile tip being cut at the same angle as was the stump so that it fitted as perfectly as possible on the oblique surface. In this case, however, the roots were placed in a horizontal position.

Experiment 1. Zea Mais—Obliquely amputated roots placed hori-zontally with the cut surface sloping upward and outward, that is with

the more shortened side below and the less shortened side above, show negative, that is upward traumatic curvature, albeit less regularly than that exhibited by similarly amputated roots placed in a vertical position.

TABLE XII—THE SUPPRESSION OF TRAUMATIC CURVATURE BY REPLACING THE AMPUTATED TIP. *Zea Mais*

| | No. of roots | Curvatures in 2 hours | | | Curvatures in 24 hours | | | |
| | | Stump | Tip | | Stump | | Tip | |
			Negative	Positive	Negative	Positive	Negative	Positive
1 mm	13	0	1	2	2	2	1	6
1·5 mm	9	0	1	0	2	0	0	5
2 mm	4	0	2	1	0	0	0	3

Thus of 23 amputated roots, 16 (69%) curved upward, 5 remained horizontal and 1 curved downward. When, however, a coleoptile tip removed by oblique section so as to fit on the end of the amputated root was placed symmetrically on the stump, only 6 out of 39 roots (15%) showed upward curvature, 14 roots remained horizontal and 19 (48%) curved downward. Thus "reheading" with the coleoptile tip causes about half the roots to curve geotropically downward, and that in spite of the tendency of the stump to curve traumatically in an upward direction. The gradient of concentration established by the oblique cut between upper and lower side is reversed. The result of amputation was to make the concentration in the upper side—containing as it does more apical tissue—greater than in the lower side. The coleoptile tip supplies more growth substance. The stimulus of gravity sets up a state of excitation in coleoptile tip and root stump. Growth substance therefore accumulates on the lower side: traumatic curvature is suppressed and

geotropic curvature takes its place. In view of these facts traumatic curvature might well be removed from the category of tropisms, since whatever other stimulus-effects wounding may have, none are involved in traumatic curvature.

(*d*) *Curvature and Growth*—The possibility that traumatic curvature depends on rate of growth was investigated by comparing the curvatures and rate of growth of pairs of similar roots amputated at a given distance from the apex, one of the pair at an angle of 45° with the long axis and the other at about half that angle, 22·5°. As the diagrams with Table XIII show, a root amputated by the more oblique section (45°) retains some apical tissue which is removed when the amputation is made at 22·5°. Therefore the gradient of concentration between the opposite sides will be higher in the stumps amputated at 45°, and the stumps will show a greater curvature than those amputated at 22·5°. On the other hand the total amount of tissue rich in growth substance removed is greater when amputation follows an angle of 22·5°. The growth of the stump amputated at that angle is less inhibited and therefore it is faster than in the stump amputated at 45°.

Amputation at 45° *and* 22·5°. *Zea Mais*—Three separate experiments were made at different times and with different sets of roots and therefore although the roots used in any one experiment, chosen because of their equality in length and rate of growth, are comparable with one another, the results of amputation at 45° or 22·5° obtained in any one experiment are not comparable with those in the other experiments. Nor indeed was it necessary that they should be since the relative curvatures at increasing distances from the tip have already been determined, and the object of these experiments was to compare the behaviour of similar roots amputated at 45° and 22·5° respectively at a given distance from the tip.

The results given in full in Table XIII may be summarized thus:—

Oblique amputation at 45° and 22·5° in 1st mm zone. *Zea Mais*

32 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature (24 hrs)	884°	637°
Total growth	124·5 mm	160·25 mm

TABLE XIII—TRAUMATIC CURVATURES OF ROOTS CUT OBLIQUELY AT ANGLES OF APPROXIMATELY 45° AND 22·5° RESPECTIVELY. *Zea Mais*

	No. of roots	6 hours			24 hours		
		No. of negative traumatic curves	Total curvature °	Total growth mm	No. of negative traumatic curves	Total curvature °	Total growth mm
1 mm zone							
	13	3	123	13·0	11	414	51·75
	10	6	101	8·75	9	284	42·25
	9	5	109	7·75	5	186	30·5
	32	14	333	29·5	25	884	124·5
	13	5	62	15·0	10	219	79·0
	10	4	69	6·5	9	199	32·5
	9	5	73	10·25	7	219	48·75
	32	14	204	31·75	26	637	160·25
1·5 mm zone							
	11	7	157	11·75	7	442	54·75
	11	5	69	10·0	9	197	32·35
2 mm zone							
	7	1	30	4·25	2	75	14·5
	7	2	29	3·75	2	33	7·0

Oblique amputation in the 1·5 mm zone.

11 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature	442°	197°
Total growth	54·75 mm	32·35 mm

Oblique amputation in the 2nd mm zone.
7 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature	75°	33°
Total growth	14·5 mm	7·0 mm

The results of the experiment in which the amputations were made in the 1st mm zone show that the more oblique amputation (45°) brings about a larger curvature and a smaller growth than does the less oblique amputation (22·5°), and it may therefore be concluded that curvature is in large measure independent of growth. The results in the other experiments in which the amputations were made in the 1·5 and 2nd mm zones respectively still show that the more oblique amputation induces larger curvature, but the growth is also greater.

Amputation in the 1st mm zone, whether at 45° or at 22·5°, removes scarcely any elongating tissues: amputation in the 2nd mm zone invades the region of active elongation, and invades it more seriously when the cut is at 22·5° than when it is at 45°; a larger portion of the most actively growing zone of the elongating region is lost to the amputated stump, and in that region the concentration of growth substance is already falling, so that the effect of the operation at 22·5° is a balance between the acceleration of growth due to the loss of more growth substance, and the reduction of total growth by the cutting away of a larger part of the growing region. That this explanation is probably true is shown by the results given in Table XIV of experiments on *Vicia Faba* with its more diffuse region of elongation.

Oblique amputation at 45° and 22·5° in 1st mm zone of *Vicia Faba* gave the following results in 24 hours:—

15 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature	829°	368°
Total growth	184·75 mm	204·5 mm

Oblique amputation in the 2nd mm zone.

20 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature	568°	255°
Total growth	171·75 mm	186·0 mm

Oblique amputation in the 3rd mm zone.

16 pairs of roots—

	Angle of amputation 45°	Angle of amputation 22·5°
Total curvature	211°	169°
Total growth	42·75 mm	35·5 mm

TABLE XIV—TRAUMATIC CURVATURES OF ROOTS CUT OBLIQUELY AT ANGLES OF APPROXIMATELY 45° AND 22·5° RESPECTIVELY. *Vicia Faba*

	No. of roots	6 hours			24 hours		
		No. of negative traumatic curves	Total curvature °	Total growth mm	No. of negative traumatic curves	Total curvature °	Total growth mm
1 mm zone							
	15	14	437	31·25	15	829	184·75
	15	11	225	27·25	15	368	204·5
2 mm zone							
	20	15	311	28·5	20	568	171·75
	20	8	101	32·0	17	255	186·0
3 mm zone							
	16	7	139	12·75	9	211	42·75
	16	6	114	10·5	7	169	35·5

Thus whereas in *Zea Mais* the greater curvature and lesser growth of the roots amputated at 45° as compared with those of the roots amputated

at 22·5° obtains only for amputation in the first millimetre zone, in *Vicia Faba* it holds good for the roots amputated in the 2nd as well as for those amputated in the 1st mm zone.   It is therefore to be concluded —as is to be expected on the growth substance hypothesis—that curvature is in a large measure independent of rate of growth.

(B)   *Positive Curvature*

It has been shown (p. 105, Table VIII) that oblique amputation of the root tip in any part of the region of response gives rise to negative curvature only and it is evident that this consistency in the direction of curvature is explicible in terms of the growth substance hypothesis.

It has now to be shown that positive traumatic curvature admits also of a like interpretation.

The experiments in semi-amputation at 1 mm from the extreme tip already recorded (p. 96) have demonstrated that roots of *Vicia Faba* show a constant negative curvature and no positive curvature even after 24 hours, but that roots of *Zea Mais* amputated at the same distance from the extreme tip though they give only negative curvatures after 6 hours show after 24 hours some positive as well as S-shaped curvatures together with a large preponderance of negative curvatures.

Semi-amputation experiments, in all respects similar to those already described except that the amputation was made at 2 mm from the extreme tip, show that the general direction of curvature is reversed.

Thus (see Table III, p. 99) semi-amputation of the root of *Zea Mais* at 2 mm from the tip results in extremely few negative curvatures of the stump ; but on the other hand it gives rise to numerous and well-marked positive curvatures of the tip.   Thus in 6 hours 9 roots and in 24 hours 26 out of 37 roots show positive tip curvature.   These tip curvatures are confined to that part of the root lying between the wound and the extreme tip.   They manifestly owe their origin to the fact that with semi-amputation at 2 mm a large part of the growing region lies apically to the section and the longer the experiment is continued the longer that part becomes. The prevention of the passage of growth substance upward beyond the section which is ensured by the mica slip leads to an accumulation of growth substance on the amputated side below the section. Growth on that side is more inhibited and the tip curves positively towards the cut.

The origin of the occasional negative tip curvatures shown in Table III must for the present remain unexplained.

That the positive curvatures are to be accounted for in the manner stated is confirmed by the results obtained with *Vicia Faba* (Table II, p. 98) in which semi-section at 2 mm gave rise mainly to negative curva-

ture. Owing to the longer elongating region it takes more time in *Vicia Faba* than it does in *Zea Mais* for the bulk of the elongating region to be transferred from the distal to the apical side of the cut. As a consequence negative largely predominates over positive curvature.

Thus (Table II, p. 98) out of 10 roots after 24 hours 7 showed negative and only 1 positive curvature. Semi-section made further back as has been shown by Snow (1924) results in positive curvature.

It is therefore concluded that negative curvature takes place when the semi-section is so situated that it hinders growth substance from reaching the region of elongation on the cut side, and positive curvature when the semi-section hinders growth substance from escaping from the region of elongation on the cut side. The S-shaped curvatures described on p. 99 must be taken to exhibit the transition from the one stage to the other.

Similarly the swellings above the cut which not infrequently occur when the root is wounded laterally must be ascribed to over growth of tissues which are cut off from normal supplies of growth substance—a conclusion which raises the question whether the frequent tumification exhibited in natural vegetation and also in plants in a pathological condition may not be due to a similar cause.

The tip curvatures recorded on p. 112 which arise in obliquely amputated and reheaded roots are evidently to be attributed to a similar cause to that operating in the semi-amputated roots. The restoration of communications remains incomplete in the reheaded roots; growth substance tends to accumulate on both sides, but more on the side containing most secretory tissue and therefore the tip curvatures, with rare exceptions, are in the positive direction. Moreover, they present striking examples of curvatures in regions in which elongation is extremely slow, so slow indeed that the appearance of the curvature suggests that it originates in a contraction of the concave side rather than in an extension of the convex side.

5—SUMMARY

Negative and positive traumatic curvature may be interpreted in terms of growth substance and the gradient of concentration of growth substance on opposite sides of a wounded root.

Inasmuch as they are the direct consequences of the asymmetry of distribution of growth substance brought about by the wound and not

a response to a specific stimulus, traumatic curvatures are not to be regarded as tropistic.

Growth substance occurs in the root of *Zea Mais* and must be regarded as a normal secretion of the tip.

The wound substance which appears on the surface of a wounded root tip is in the first instance and mainly an exudation of that preformed in the intact root.

The receptor for the stimulus of gravity appears to extend from the tip through the vegetative apex (formative region), into a part at least, if not the whole, of the elongating region.

The secretion of growth substance is localized. It is most active in the extreme tip, falls off rapidly (in *Zea Mais*) in the 1st mm zone, and less rapidly in the 2nd mm zone, beyond which little if any secretion occurs.

Negative traumatic curvature is an harmonious curvature of the root as a whole, due to the wound preventing growth substance from reaching the side of the elongating region above the wound.

Positive curvature is a tip curvature, *i.e.*, one confined to the part of the root in front of (apical to) the wound. It is due to the wound preventing the escape of growth substance and thereby bringing about an increase in concentration in the part of the root apical to and on the same side as the wound.

Growth and curvature bear no quantitative relation to one another; the one being related to the concentration of growth substance in the growing region, the other to the gradient of concentration between its opposite sides.

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632.8

On the Interactions of two Strains of a Plant Virus; Experiments on Induced Immunity in Plants

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[PLATES 14–16]

INTRODUCTION

The nature of the causative agent of virus diseases is obscure. A great deal of information on the reactions of the agent and on the symptomatology of these diseases has, however, been accumulated, with a view to bringing to the solution of the main problem the largest available amount of detail. While there is no irrefutable argument in support of either the organismal or the non-organismal hypothesis, it has become clear, as the investigations have progressed, that the viruses of both plant and of animal diseases possess many of the qualities usually associated with organisms. The existence of strains in the animal viruses is now generally accepted, though less information has been available regarding this aspect of the plant viruses. The purpose of the present paper is to direct attention to the presence of strains in what was previously thought to be a single virus, and to the probability of similar conditions obtaining in other viruses. The interactions of these strains, one with another, and with other viruses have been examined and are also described and discussed.

It is shown that four types of interaction between different plant viruses can be recognized and these types are examined in some detail.

MATERIALS AND METHODS

The virus which was used in the study of strains was that of "Aucuba" or Yellow Mosaic of tomato. This virus has been intensively studied in this laboratory for some years and there is good evidence for supposing that it is identical with the *Tobacco Virus No.* 6 of Johnson, the causative agent of Yellow Mosaic of tobacco. Yellow Mosaic of tomatoes has been known in this country for some years, and was first described by Bewley (1924), the causative agent being studied in some detail by Henderson Smith (1928). The viruses were studied in their host plants

tomato (*Lycopersicum esculentum* var. "Kondine Red"), tobacco (*Nicotiana tabacum* var. "White Burley"), *Nicotiana glutinosa*, and *Solanum nodiflorum*.

Henderson Smith described the virus as being "possibly identical with Johnson's *T.V. No.* 6 which differs from that of ordinary tomato mosaic in the brilliance and intensity of its leaf symptoms, but in other respects is indistinguishable from it by the characters investigated." These characters were its longevity, its heat resistance (it is not destroyed by exposure to 80° C for 10 minutes), its resistance to alcohol up to 90% and to other chemicals, and its infectivity in diluted juice. The symptoms of the disease are described as follows: "In extreme cases almost the whole of the surface is pale yellow to white with here and there small islets of intense dark green which stand up as small blisters. In less extreme cases, the green areas are larger, but as a rule the area of white or pale yellow is greater than the green area . . . Scattered over the leaf are patches of white and patches of yellow, usually sharply delineated, but sometimes shading into neighbouring areas, irregular in shape and size, often angular and occurring in all parts of the leaf . . . The plant is not killed . . . There is no necrosis in tomato, . . . There is little tendency to extreme malformation though quite definite "fern-leaf" has been noted on plants growing rather slowly, *e.g.*, in the autumn . . ."

To this description some further details may now be added. The virus attacks a wide range of hosts, particularly among the *Solanaceæ* and in many it produces symptoms very similar to those described for tomato. Further, in some hosts, notably *S. nodiflorum*, it induces the formation of "X" bodies in the cells. The large spherical inclusion bodies induced by this virus in *S. nodiflorum* are characteristic of the disease, and may be used for diagnostic purposes, being readily distinguished from the smaller inclusion bodies typical of other viruses in this host.

From the observations made in this laboratory on Yellow Mosaic of tomato since the publication of the paper cited above, it was concluded that the disease was characterized by two main groups of symptoms. In summer, when plants are growing well and light intensity is high, the symptoms were as described above. In winter, on the other hand, and when the growth of the tomato plants was slow, the chlorosis was much less marked and was often indistinguishable from the indeterminate mottle which is characteristic of the tomato Mosaic caused by *T.V. No.* 1 of Johnson (1927). It was therefore assumed that there were "summer" symptoms, formed in conditions of high light intensity, and "winter"

symptoms which occur when the light intensity is low and the growth of the plants reduced.

It was not until the spring of 1933 that evidence of the existence of strains in the virus presented itself. It was then noticed, after a spell of particularly sunny weather, that individual plants of a group of tomatoes inoculated with a culture of Yellow Mosaic virus, which had been kept in our glasshouses since 1925, showed unusual symptoms. Some were only faintly mottled as is typical of very mild " winter " symptoms, while others showed the most marked chlorosis with intensely bright yellow areas on the leaves; the majority occupied an intermediate position as regards symptom intensity. In this instance, at least, light intensity was evidently not the controlling factor in symptom expression, since three distinct groups of symptoms were obtained under the same environmental conditions. The existence of different strains of the virus, possibly an attenuated and a more virulent form, as have been shown to exist in *T.V. No.* 1, was, therefore, suspected. With a view to examining this possibility inocula were prepared from single plants showing the typical symptoms of each group and were inoculated into healthy tomato seedlings. After an appropriate interval symptoms of the same types were developed in the inoculated plants. Cultures were made from the two extreme groups, the mild type of symptoms being for convenience called " green " Mosaic and the more severe types being called " yellow " Mosaic. These were the materials with which the experiments now to be described were carried out.

The " Green " Material—To ensure that no contamination of the stock material had taken place, the juice of diseased tomato plants showing the " green " type of symptoms was submitted to the standard treatment *in vitro*, to determine the physical properties of the virus responsible for the development of symptoms. The properties were those characteristic of the virus of classical " Aucuba " material, *i.e., T.V. No.* 6. This did not preclude the possibility of the virus being *T.V. No.* 1, however, which has identical properties *in vitro* with *T.V. No.* 6. A simple test with tobacco plants is sufficient to distinguish between these two viruses, since *T.V. No.* 6 normally causes the formation of necrotic spots on the rubbed leaves of tobacco, while *T.V. No.* 1 causes only faint chlorotic spots or no visible symptoms on the rubbed leaves. Two groups of young tobacco plants were, therefore, inoculated one with *T.V. No.* 1 juice, the other with juice from a plant with " green " Mosaic. In the former group there were no local necrotic lesions on the rubbed leaves, the first symptoms being the systemic chlorosis typical of tobacco Mosaic, while in the

latter group local necrotic lesions appeared on the rubbed leaves on the third day after inoculation. It is clear, therefore, that the " green " Mosaic virus is not identical with *T.V. No.* 1 and for convenience it is designated subsequently as A.G. virus in this paper.

After this point had been established, serial inoculations of the A.G. material were made at intervals of three or four weeks into groups of tomato plants, care being taken to avoid contamination and to examine the plants for the appearance of the usual symptoms of tomato Yellow Mosaic. After a large number of such transfers it was found that the A.G. virus was quite consistent in its action in the host plant and a series of experiments with other hosts was set up. The symptoms found in the different hosts were as follows:—

(*a*) *In Tomato*—A faint generalized mottle with little leaf distortion and little stunting of the plant. Symptoms appear some 10 to 15 days after inoculation.

(*b*) *In Nicotiana glauca*—No necrotic local lesions (inoculations were made by the pin-prick method since there is an almost complete absence of hairs on the leaf of this plant). A faint mottle visible on the upper leaves of some plants a few weeks after inoculation, but, more generally, no apparent symptoms.

(*c*) *In Solanum nodiflorum*—No necrotic local lesions—and systemic symptoms a faint mottle with little leaf distortion or stunting of the plant. Symptoms appear some 12 to 15 days after inoculation. The inclusion bodies in the infected hair-cells are quite typical of " Aucuba " or Yellow Mosaic of tomato.

(*d*) *In Nicotiana tabacum* (var. " White Burley ")—Marked necrotic local lesions on the rubbed leaves followed by a systemic chlorosis with or without necrosis (occasionally causing death of young plants).

(*e*) *In Nicotiana glutinosa*—Marked necrotic local lesions on the rubbed leaves. No systemic infection.

(*f*) *In Zinnia sp*—No apparent local lesions on the rubbed leaves. Faint mottle on older leaves some 14 days after inoculation.

The " Yellow" Material—The " yellow" material was treated in a similar manner. Serial transfers from group to group of tomato plants led to the conclusion that this virus gave consistent results in this host. It was, therefore, designated A.Y. virus for convenience. In all its reactions *in vitro* this virus is identical with the A.G. virus above described and

in the next section evidence is adduced to show that they are, in fact, strains of the same virus, viz., *T.V. No.* 6. It is further suggested, as a result of observations detailed later, that the disease as it occurs in the field is usually caused by a mixture of these two strains.

The symptoms induced by the A.Y. virus in the same host plants as were used for the A.G. virus are as follows:—

(*a*) *In Tomato*—A bright yellow mottle with some leaf distortion and stunting of the plant. Symptoms appear from 5 to 20 days after inoculation. In bright sunny weather the incubation period is shorter.

(*b*) *In Nicotiana glauca*—No necrotic local lesions—inoculations were made by the pin-prick method since there is an almost complete absence of hairs on the leaf of this plant. Bright yellow symptoms appear on the young leaves after some 10 days. The mottle often takes the form of concentric rings. There is little distortion or stunting of the plant or leaves and no necrosis.

(*c*) *In Solanum nodiflorum*—No necrotic local lesions. Occasionally chlorotic local lesions on leaves rubbed when young. The systemic symptoms, as in tomato, are a bright yellow mottle. There is no necrosis. The symptoms appear 7 to 20 days after inoculation. The inclusion bodies in the infected hair-cells are quite typical in this mosaic.

(*d*) *In Nicotiana tabacum* (var. " White Burley ")—Marked necrotic local lesions developed on the rubbed leaves, followed by a systemic chlorosis with or without necrosis (occasionally, in the former instance, causing the death of young plants). The symptoms induced by the A.Y. virus strain in this plant are very similar to those caused by the A.G. virus strain.

(*e*) *In Nicotiana glutinosa*—Marked necrotic local lesions on the rubbed leaves. No systemic infection.

(*f*) *In Zinnia sp.*—Occasional chlorotic local lesions on the rubbed leaves. Bright systemic mottle irregularly on younger leaves of plant after some 10 days incubation.

A series of photographs of the plants, Plates 14–16, illustrate clearly the marked differences in the symptom-picture induced by the two strains of the virus.

The Identity of the Virus Strains

A detailed examination of the two strains was made to discover whether they are actually strains of the same virus or two closely related viruses.

Many of the symptoms induced by the A.G. virus, for example, might equally well have indicated the presence of a mixture of two viruses, viz., *T.V. No.* 1 and *Tomato Streak Virus No.* 1. Those two viruses have reactions *in vitro* identical with those of *T.V. No.* 6 and are separable from it only through differential hosts. *T.V. No.* 1 induces no necrotic local lesions in tobacco but only a systemic chlorosis, while *T.S.V. No.* 1 induces local necrotic lesions and no systemic chlorosis. Should systemic symptoms occur with the latter virus, they take the form of severe necrosis, which often kills the plant. If, therefore, a mixture of the two were inoculated into tobacco, it would induce local necrotic lesions, followed by a mild systemic chlorosis. This is similar to the symptom-picture of the A.G. mosaic. A second inoculation, from the upper leaves of tobacco plants infected with a mixture of *T.V. No.* 1 and *T.S.V. No.* 1, made into tobacco, would serve to demonstrate the absence of *T.S.V. No.* 1 in those upper leaves, since no local necrotic lesions would result. Inoculations into tobacco, from the upper leaves of tobacco plants infected with A.G. virus, indicated, by the development of local lesions in the rubbed leaves, that this was not a mixture of the *T.V. No.* 1 and the *T.S.V. No.* 1 viruses.

Further evidence on this point was furnished by a study of the leaf hairs of *S. nodiflorum*. In the cells of this plant are developed the characteristic inclusion bodies of the Yellow Mosaic disease. These have been described in some detail by both Henderson Smith (1930) and Sheffield (1931), working in this laboratory. Inoculations of A.G. virus material were made into one group of *S. nodiflorum* plants while inoculations of a mixture of *T.V. No.* 1 and *T.S.V. No.* 1 were made into a second group. After a fortnight, the inclusion bodies in the cells of both sets of plants were examined. The cells of the leaf-hairs of the plants inoculated with the A.G. virus contained the usual inclusion bodies (" X " bodies) typical of the disease, while those of the other plants had inclusions of a different type, characteristic of *T.V. No.* 1.

Infections from the upper leaves of the plants infected with the mixed viruses were made into young tobacco plants. It was found that both viruses had multiplied in the tissues of the *S. nodiflorum* plants as the inoculated leaves of the tobaccos developed necrotic local lesions typical of the *T.S.V. No.* 1, while the later appearance of typical systemic chlorosis indicated the presence of *T.V. No.* 1.

All the available evidence, therefore, points to the view that these two viruses A.G. and A.Y. are, in fact, strains of the virus known as *T.V. No.* 6.

A series of experiments were set up in which the two strains were mixed together and inoculated into young tomato plants. Both are easily inoculated by rubbing the host plant with infected juice and both multiply readily in the tissues; there is no evidence that one is more virulent than the other.

The question of concentration of the viruses does, however, arise in this connection and an examination was made of the amount of virus present in the tissues of similar tomato plants inoculated with each of the strains separately and with a mixture of both strains. A very fair measure of the amount of the virus present may readily be made by counting the necrotic lesions which follow the inoculation of known amounts of extracted juice on the leaves of *N. glutinosa*, in which plant, as has been shown (Chester, 1933) both strains induce the same symptoms, viz., necrotic local lesions on the rubbed leaves (Caldwell, 1933). When similar amounts of diseased tissue, macerated in water, were inoculated on to the leaves of *N. glutinosa*, no difference was found in the concentration of the virus in tomato plants inoculated with the A.G. strain, the A.Y. strain, or with a mixture of both.

In the first group of experiments on inoculation with the mixture of the two strains A.G. and A.Y., the two materials were mixed in equal proportion and inoculated into young tomato plants. After an appropriate interval symptoms of normal Yellow Mosaic appeared on the young leaves. The symptoms appeared to be intermediate in intensity between those of the A.G. form and of the A.Y. form. Neither strain was, apparently, able completely to inhibit the development of the other under the environmental conditions obtaining during the experiment.

When mixtures in which one of the components was in considerable excess were used as inocula the symptoms developed in the tomato plants tended, in the main, to approximate more nearly to those associated with the major component.

The symptoms caused by the A.G. strain clearly could not be detected in a plant previously inoculated with the yellow strain, but the converse does not hold. The intensity of the symptoms caused by the yellow strain is so much greater than that of the symptoms of the green that they can be easily recognized in the presence of the latter. A series of experiments was therefore, set up to discover the effect of inoculating a plant already infected with A.G. virus with the A.Y. strain.

A group of tomato plants were inoculated with the A.G. strain and

after a fortnight showed symptoms typical of this virus. Thereafter, further inoculations of A.Y. material were made into the same plants, but no symptoms of Yellow Mosaic were developed. This has been repeated many times and so far, 18 months after the isolation of the original cultures of the strains, no instance has occurred in which the A.G. strain has failed to immunize plants against the A.Y. strain. This immunity is apparently complete in that it is not possible to recover from plants which have been inoculated with the A.G. and then with the A.Y. strain any trace of the A.Y. strain. Many attempts have been made involving a large number of plants and the immunity is not only expressed in the suppression of symptoms, but also in the prevention of development of the second strain within the tissues.

This immunity is rapidly set up in the tissues as attempted infection of tomatoes with the A.Y. strain three or four days after inoculation with the A.G. strain has been consistently unsuccessful. If the second inoculation be made into the younger leaves before the first virus has left the inoculated leaf, which is usually some 48 hours or so after inoculation, then symptoms of the " mixed " type are usually found.

Immunity against the A.Y. Strain induced by the A.G. Strain in *Zinnia sp.*

The complete immunity against the A.Y. strain which is induced in tomato by a previous infection with the A.G. strain of virus is found also in *Zinnia sp.* Groups of plants were inoculated with A.G. strain and were allowed to develop the very mild symptoms characteristic of the disease. Thereafter one-half of the plants were inoculated with A.Y. strain as were also a group of healthy controls. The controls all developed marked symptoms of Yellow Mosaic after some 10 days, while the other plants did not. At the end of a fortnight the top leaves of plants from each group were inoculated into groups of tomato in which the tissues from the Zinnias infected with A.G. strain produced the usual green symptoms, as also did those which had been inoculated with both strains, while the leaves of the plants with the yellow strain produced the usual marked symptoms of Yellow Mosaic. In *Zinnia*, therefore, there is apparently complete immunity against the A.Y. strain induced by the A.G. strain of virus.

The Appearance of Summer and Winter Symptoms of Yellow Mosaic

Mention has been made of the fact that the A.Y. strain appears to have a variable incubation period, depending on the light intensity at

the time of inoculation. During the winter months plants in our glass-houses were kept under 1000-watt lamps at a distance of 3 to 4 feet for some 6 hours each night to make up for the deficiency of light during the day. Inoculations were made with both strains and tomatoes so infected showed the symptoms typical of the appropriate strain. The " green " symptoms were no more intense under the lights than they were in plants grown under normal conditions.

The fact that the two strains retain their identity over long periods was shown by a set of experiments on filtration. The juice of plants infected with the A.Y. and with the A.G. strains was passed through filter paper impregnated with fuller's earth and then through L1 and L3 Pasteur-Chamberland filters. The sterile juice so obtained was kept for 6 months in sealed tubes under aseptic conditions, and the juice then inoculated into tomato and *N. glutinosa* plants. The A.G. strain induced the formation of " green " symptoms, the A.Y. strain of " yellow " symptoms—similar to those on the plant from which the virus material had been obtained. Another sample of each juice was kept for a year under similar conditions and gave similar results on inoculation after that interval.

The existence of these two strains in the virus of Yellow Mosaic, and the possibility that there may be more strains not yet identified, offer a probable explanation for the differences in the summer and winter symptoms induced by this virus on tomato. The green strain appears to have the shorter incubation period under winter conditions while the yellow has the shorter under summer conditions. If one assumes that the virus as obtained from the field has some of these and probable other strains present in it, then, in the dull cold conditions of winter, the green will tend to spread more rapidly than the yellow strain, giving the winter symptoms, whereas the reverse would hold in summer. This phenomenon has, in fact, been observed. A stock of Yellow Mosaic had been kept in our glasshouses in tomato plants grown, as a precautionary measure, under muslin cages; at various intervals, material was taken from these plants and used for experimental purposes. In the spring of this year it was noticed that the stock plants had ceased to show the bright yellow symptoms characteristic of the disease and were only faintly mottled—they did show symptoms characteristic of the A.G. strain. When inoculations were made from these plants into healthy plants of tomato, and secondary inoculations with A.Y. strain were made after an interval of 10 days, the yellow symptoms characteristic of the second strain did not appear in any of the plants. It is suggested that the effect of keeping the plants under muslin was to maintain their metabolism at the " winter "

rather than the " summer " level and that, in consequence, over a long series of transfers, the " green " strain of virus had been encouraged at the expense of the " yellow." In the earlier samples enough of the " yellow " strain had been left to allow of its multiplication under normal summer conditions.

In some virus diseases (Salaman, 1933) isolated portions of the infected plants appear to contain different components of the virus complex. The virus under discussion does not appear to belong to this group. The tiny yellowish flecks which occur infrequently on the leaves of plants infected with A.G. strain have been punched out of the leaves with a punch made from narrow-bore brass tubing, to preclude the removal of other portions of the leaf at the same time. Inoculations with these isolated portions have invariably been followed by the development of typical symptoms of the A.G. strain in tomato plants. Similarly, inoculations with portions of the green tissue from leaves infected with A.Y. strain are always followed by the development of the normal symptoms of the A.Y. strain. Specific cells of the host plants infected with a mixture of these two strains apparently become infected with one or other virus-strain, but there is no evidence that substantial portions of the lamina are infected with any one strain to the exclusion of the other.

THE IMMUNITY AGAINST YELLOW MOSAIC VIRUS INDUCED BY OTHER
VIRUSES

When the complete immunity to the A.Y. strain, induced in plants by infection with the A.G. strain of tomato Yellow Mosaic virus, had been clearly established an examination was made of the immunity, if any, induced by other viruses against the same virus. The viruses examined and the results obtained are detailed in the succeeding paragraphs.

(a) *The Interaction of Tobacco Mosaic and Tobacco Yellow Mosaic Viruses*—The first reaction examined was that of *T.V. No.* 1 with the strains A.Y. and A.G. This virus is the agent of the commonest of all tobacco mosaics and, as has been seen, differs from that of tobacco Yellow Mosaic only in the intensity of the symptoms produced in tomato, and in some other hosts, being indistinguishable from it as regards reactions *in vitro*. There may, therefore, be some close affinity between these two viruses, especially as there have been suggestions that a yellow form of tobacco Mosaic may arise in plants infected with *T.V. No.* 1 (Jensen, 1933). A group of six tomato plants were inoculated with *T.V. No.* 1 and after a week they showed marked symptoms of ordinary Mosaic of tomatoes. They were then inoculated with the A.Y. material

as were also another group of tomatoes of the same age, which being previously healthy served as controls. A few days later the control plants developed the typical symptoms of Yellow Mosaic, while no trace of this disease was evident in the doubly inoculated plants. After a fortnight, which is three times the maximum incubation period for Yellow Mosaic in summer, the top leaves of the infected plants were removed and macerated with water.

A simple diagnostic difference between these two viruses is the appearance of necrotic local lesions on the leaves of tobacco rubbed with *T.V. No.* 6 (the agent of Yellow Mosaic). The macerated material prepared from the tops of the doubly-inoculated plants was therefore rubbed on to leaves of tobacco plants. No necrotic local lesions appeared on any of the inoculated leaves. Systemic symptoms of ordinary tobacco mosaic did develop, however, after an appropriate interval.

In order to ascertain whether *T.V. No.* 1 might have an inhibitory effect on *T.V. No.* 6 which might prevent the appearance of symptoms on the rubbed leaves of tobacco, in the presence of the first virus, a mixture of juices containing the two viruses was inoculated on to leaves of tobacco. Normal necrotic local lesions developed within 3 days. It is clear, therefore, that the presence of *T.V. No.* 1 does not prevent the appearance of the necrotic lesions induced by *T.V. No.* 6.

In the tops of the plants, which had been doubly inoculated as described above, no multiplication of *T.V. No.* 6 had taken place. The immunity against *T.V. No.* 6 induced in the tomato by *T.V. No.* 1 is apparently complete, and not only are no symptoms produced, but the development of the virus is also inhibited. The A.Y. virus was clearly available to the experimental plants, as there was 100% infection of Yellow Mosaic in the controls, in these and in other experiments.

Similar groups of experiments were set up with the A.G. strain and *T.V. No.* 1. Tomato plants were inoculated with the latter virus and, after symptoms had developed with the former. After a fortnight or three weeks the tops were removed and macerated in water. No attempt was made to distinguish symptoms indicative of A.G. virus against those of *T.V. No.* 1 as the A.G. virus symptoms are less conspicuous than the others. Four groups of tobacco plants were inoculated, the first with the material from the top leaves of the tomato plants above mentioned, the second with a mixture of juices containing *T.V. No.* 1 and A.G. strain virus, the third with *T.V. No.* 1 material alone and the fourth with A.G. strain material alone. No necrotic local lesions were formed in the first and third groups, but the normal lesions characteristic of *T.V. No.* 6 were found in the leaves of the plants of the second and fourth groups.

T.V. No. 1, therefore, induces in tomato plants as complete an immunity against A.G. strain virus as it does against the A.Y. strain of the same virus. No development of either strain, apparently, takes place in tissues infected with *T.V. No.* 1.

(*b*) *The Interaction of the Virus of Valleau's Ring Mosaic and the A.Y. Strain*—The virus of Valleau's Ring Mosaic (Valleau and Johnson, 1930) causes a disease of tobacco, which as its name suggests, is characterized by the appearance on the leaves of chlorotic rings. Under the conditions in our glasshouses the disease symptoms in tomato are not very well marked, consisting of necrotic lesions on the stems, leaves, and petioles. Very occasionally the necrosis may be severe, the condition which is common, apparently, in America.

Groups of tomato plants were inoculated with the virus of Valleau's Ring Mosaic (subsequently called V.R.M.) and after some 10 days when the first symptoms of the disease had appeared they were inoculated with the juice containing the A.Y. strain. No symptoms of the Yellow Mosaic had appeared on the plants after 4 weeks, although the inoculated controls showed the usual symptoms on the fifth or sixth day. Occasional chlorotic areas did appear on some of the plants some 6 weeks after the second inoculation.

The V.R.M. virus differs from that of Yellow Mosaic in that the latter survives heating at 80° C for 10 minutes while the former is destroyed at that temperature. The upper leaves of plants inoculated with both viruses were removed 4 weeks after the second inoculation, were macerated with water and were inoculated on to the leaves of *N. glutinosa* plants. Necrotic local lesions developed on the rubbed leaves, but since both viruses induce these symptoms, it is not possible to separate them in this way. Another portion of the same juice was heated at 80° C for 15 minutes, was cooled, and inoculated on to other leaves of *N. glutinosa* plants. In this instance no necrotic lesions appeared. The A.Y. virus had therefore not reached the upper leaves of the doubly-inoculated plants in less than 4 weeks. As has been noted, some of the doubly-inoculated plants did, as they grew older, show symptoms of Yellow Mosaic and from the upper leaves some A.Y. virus was recovered. This has been tested out on *N. glutinosa* on tobacco and on tomato. The concentration of virus in them, however, was very low and multiplication of the A.Y. virus is much reduced in these plants in which it is not completely inhibited by V.R.M. virus.

(*c*) *The Interaction of " Streak " Virus with the A.Y. Virus*—There occurs in the commercial glasshouses in this and in other countries a

disease of tomatoes known as " Streak." One form of this disease is caused by a single virus known as *Tomato Streak Virus No.* 1 (*Tom. S.V. No.* 1), and it is with this virus that these experiments were carried out. This virus causes two types of disease in the tomato; one is necrotic and is characterized by the presence on the leaves and petioles of necrotic lesions, often scar-like (hence the name " Streak "). The other type is purely chlorotic and is not unlike, in symptoms, the diseases caused by *T.V. No.* 1, viz., ordinary tomato Mosaic. Tissue from plants showing either form of the disease may, on inoculation, induce in healthy tomato plants either form of the disease in different plants of the same batch, though what factor conditions the type of disease symptoms which will develop is not as yet known.

Groups of tomato plants were inoculated with " Streak " virus and after some 10 days, when the symptoms of the disease had appeared, with A.Y. virus. Groups of healthy plants were also inoculated with the A.Y. strain. After a fortnight, many days after the inoculated controls showed symptoms of Yellow Mosaic, the doubly-infected plants were examined and were found to show no symptoms of Yellow Mosaic.

Tom. S.V. No. 1 and the A.Y. strain may be distinguished by the fact that the former induces in tobacco necrotic local lesions followed usually by no systemic infection, or occasionally by systemic necrosis, while the latter, as has been seen, induces necrotic local lesions followed by systemic chlorosis. The appearance, therefore, in inoculated tobacco plants of necrotic local lesions, would not distinguish between the two viruses, whereas the subsequent development or non-development of systemic chlorosis would.

The upper leaves of plants which had been inoculated with both viruses were, therefore, macerated in water and the extracted juice was inoculated on to the leaves of young tobacco plants. After an interval of 4 days, the rubbed leaves showed marked necrotic lesions while after 7 to 10 days, a proportion of the plants showed systemic chlorosis, with or without necrosis, and when juice from their upper leaves was inoculated into young tomato plants Yellow Mosaic symptoms appeared. The effect of the presence of the " Streak " virus was, therefore, to inhibit the appearance of the symptoms of Yellow Mosaic in the doubly-inoculated plants, though the presence of *Tom. S.V. No.* 1 in the tissues does not, apparently, completely inhibit the development of A.Y. virus inoculated secondarily.

(*d*) *The Interaction of Potato " X" Virus with the A.Y. Strain*—The potato Mosaic Virus " X " has been studied at some length by various workers

and numerous strains have been isolated. It is readily juice transmitted and in the tomato induces a disease " Spot Necrosis " which is a systemic chlorosis, with or without tiny necrotic spots. When this virus is inoculated into tomatoes together with a tobacco virus the disease caused is a very severe necrosis—" Experimental Streak " (Dickson, 1923). The " X " virus is less stable than are the tobacco viruses and is destroyed by heating the diseased juice at 70° C for 10 minutes. Groups of tomato plants were inoculated with " X " virus and after 10 days, when the disease symptoms were clearly defined, the plants were inoculated with the A.Y. strain. After 5 days every plant developed the most marked necrosis characteristic of " Experimental Streak," which indicated, not only that the " X " virus had in no way inhibited the development of the A.Y. strain in the infected tissues, but that the two viruses together induced a more severe disease than either alone. Material from the tops of the plants was macerated with water, filtered, heated at 80° C for 15 minutes and inoculated into young tomato plants where, after an appropriate interval, the typical symptoms of Yellow Mosaic developed. In this instance, the presence of the first virus in the tissue had not acted as a preventive, but had, in fact, resulted in the development of more severe symptoms after the second infection than would have been induced by either of the viruses acting alone.

Similar results were obtained when the A.G. strain was used in conjunction with the " X " virus.

(e) The Interaction of " Spotted Wilt " Virus with the A.Y. Strain— The virus of the tomato disease " Spotted Wilt " is unlike any of the other viruses used in these experiments as it is destroyed by exposure to a temperature of 45° C for 10 minutes, whereas the other viruses are relatively thermo-stable. The other viruses are also fairly resistant to ageing, while the " Spotted Wilt " virus is inactivated after a few hours' exposure at room temperature. The symptom picture in the tomato of this disease is quite unlike that of Yellow Mosaic, being bronzing of the leaves, marked stunting of the plant, and some necrosis.

Groups of tomato plants were inoculated with this virus and showed signs of infection within 5 days. The effect of the first infection was so marked and the disease symptoms so severe that the plants made practically no growth, having been inoculated as fairly young plants. For this reason they were allowed to grow for some 5 weeks before an inoculation with A.Y. virus was made. Symptoms of Yellow Mosaic appear on the doubly-inoculated plants rather slowly, the delay being due, it is believed, to the slow growth of the plants rather than to the inhibition of

the development of the virus by that of " Spotted Wilt." The upper leaves of the plants were macerated with water and used as an inoculum. This juice was left overnight on the laboratory bench and was heated at 70° C for 10 minutes before inoculation into young tomato plants. Either of these treatments is sufficient to destroy the " Spotted Wilt " virus. The inoculated tomato plants developed typical symptoms of Yellow Mosaic, showing that the two viruses in this instance develop independently and that each induces the formation of the appropriate disease symptoms.

(*f*) *The Interaction in Tobacco of Ring Spot Virus and the A.Y. Strain*— The virus of Ring Spot disease of tobacco does not readily infect the tomato, which can only be infected with this disease by grafting (Price, 1933). The virus is easily transmitted by rubbing to tobacco, where it induces the development of the chlorotic rings which give the disease its name, with the subsequent development of some necrosis (Price, 1933). A series of tobacco plants were inoculated with Ring Spot virus kindly given to me by Dr. J. M. Birkeland. After the symptoms had become well marked on the inoculated leaves, the leaves were rubbed with juice containing A.Y. virus. In 3 days marked necrotic lesions appeared on the rubbed leaves and there was not the slightest evidence that the presence of Ring Spot virus affected the development of the A.Y. strain except in so far as the reduction of growth in the infected plants tended to make the symptoms induced by the A.Y. virus less marked than they would have been in normal infected plants. Apart from that, the Ring Spot virus appears to have no specific effect on the virus of Yellow Mosaic. The presence of active A.Y. virus was demonstrated by inoculating some of the upper leaves of the tobaccos into young tomato plants into which Ring Spot virus cannot be introduced by juice inoculation. In these plants, typical symptoms of Yellow Mosaic readily developed.

DISCUSSION

A considerable volume of work on plant virus diseases and on the nature of the causative agent has established the following facts :—

(*a*) There exist virus complexes, which, especially in the potato diseases, can be separated more or less readily into their component viruses. This may be effected by passage through " filter plants " by heat treatment or by making use of some other differences in the host and *in vitro* reactions of the viruses. Different mixtures of these viruses may give rise to different disease symptoms in the appropriate host plants.

(*b*) The virulence of a virus may sometimes be increased or diminished by passage through an unusual host or by special treatment *in vitro*. Passage through *Chenopodium murale*, for example, attenuates the virus of Curly-Top disease of sugar-beet, while subsequent passage through *Stellaria media* restores its virulence. Johnson (1928), Kunkel (1934), and others have shown that the exposure of tobacco plants to a temperature of 35°–36° C for some days after inoculation not only seriously affects the expression of the symptoms produced, but also attenuates the viruses of the tobacco Mosaics since these viruses are recovered in a much weakened form.

The separation of mixtures of viruses into their components and the alteration in virulence of any given virus must not be confused with the isolation of separate strains of the same virus.

Hitherto there has been little evidence whether or not a particular virus might occur in different strains. McKinney (1926) has reported that the small yellow spots which appear on tobacco after infection with tobacco Mosaic give rise on isolation to a Yellow Mosaic as against the green type from which the original inoculation was made. The green symptoms, he found, could suppress the yellow, and if the virus which caused the yellow symptoms were in high dilution the yellow spots did not appear. It is not clear whether this is a case of segregation of two viruses or of separation of two strains of the same virus. Jensen (1933) has recently reported a similar group of experiments with tobacco Mosaic virus (*T.V. No.* 1). Bennett (1932) in a recent paper suggests that the different Mosaic diseases of raspberry may be due to strains of the same virus which have different degrees of virulence. Cooley (1932) has found, also in raspberry, that there are two Streak diseases, which, differing in one symptom only, are similar in others and appear to be closely related. Storey and McClean (1930) described strains of different virulence of the virus of " Streak " disease of maize. More recently Salaman (1933) has published evidence of the existence of at least two strains in the X virus of potato Mosaic which causes a distinct Mosaic in tobacco. He found that there were apparently different strains in the yellow and in the green areas of the infected tobacco leaf, and that the " green " strain induced immunity against the " yellow," if inoculated first into tobacco or *Datura stramonium*.

The general papers of Chester (1933) summarize the present position with regard to immunity in plants. A considerable portion of these papers is devoted to a consideration of immunity to virus diseases and a comprehensive survey of the available literature is made.

While this paper was in course of preparation Kunkel (1934) published

a paper in which he describes some experiments with strains of Yellow Mosaic virus (from the stock kept in this laboratory), these strains being isolated and studied in *Nicotiana sylvestris* plants. He considered one of the strains an attenuated form of the other, and found that the attenuated virus immunized plants against the virulent form, or against ordinary tobacco Mosaic virus (*T.V. No.* 1). The second virus did not appear to multiply in the protected tissues. Cucumber Mosaic virus or tobacco Ring Spot virus did not induce immunity against the Yellow Mosaic virus.

The author's experiments establish the fact that the virus causing Yellow or Aucuba Mosaic of the tomato does exist in at least two strains, which cannot by any known means be changed, the one into the other. Evidence has been adduced to show that the one cannot be considered as an attenuated form of the other, as no return of virulence has been induced by frequent passage through susceptible host-plants.

The experiments further show that inoculation of the plant with one of these strains prevents the multiplication of the other strain in the tissues of the infected plant, and consequently that complete immunity against one strain is induced by infection with the other.

This observation has been extended to other viruses and it has been found that four types of interaction may be recognized—

(*a*) The first interaction consists of the induction of complete immunity to a second virus by infection with a first. In this type the second virus apparently does not multiply in the host tissues and none of the second virus can be recovered from the inoculated plant.

(*b*) The second type of interaction is found with those viruses, the first of which apparently confers some degree of immunity against the second, the symptoms associated with the second virus not developing in the inoculated plant, or the development of the second virus being very much reduced by the presence of the first.

(*c*) The presence of a virus in the tissues of a plant may actually increase the severity of the symptoms induced by a second virus, making the disease caused by the two viruses more severe than that caused by either virus alone.

(*d*) The presence of a virus in the tissues of a plant may interfere with the development of a second virus insofar as the reduction in the growth of the host plant reduces symptom expression. The two viruses seem to multiply together in the tissues without mutual interference.

Some general considerations arise from this discussion. The presence of strains in plant viruses is probably much more general than was previously believed and it may be that some of the present anomalies in

symptom expression may be due to the unsuspected presence of strains, one of which induces one type of symptom, the other another. These may occur in differing proportions under certain environmental conditions. Certainly, the different effect of summer and winter conditions is much less marked with plants infected only with the A.G. strain or the A.Y. strain of *T.V. No.* 6 than it was with the original Yellow Mosaic virus, which was presumably a mixture.

The problem of the immunity against one strain of virus induced in plants by an infection with another strain is of great interest from the point of view of the light its solution would throw on the nature of the virus. The amount of virus in any given tissues must be comparatively small, having regard to the size of the virus particle. The virus of Yellow Mosaic, with which this paper largely deals, has a diameter of approximately 40 μμ (McClement and Smith, 1932) and this refers to the size of the virus unit necessary to induce a necrotic lesion on the leaves of *N. glutinosa*—not necessarily to the size of a single virus body.

The number of these units per cubic centimetre in any given juice has been shown by the author (Caldwell, 1933) to be comparatively small. There must be, therefore, either only a few foci in the tissues at which the virus can multiply, or some reaction of the plant to the virus infection which prevents the multiplication of a second virus sensitive to the presence of the first. The second explanation seems the more probable since the multiplication of two mutually tolerant viruses in a tissue could only be accounted for, on the first hypothesis, by the assumption that different viruses had different foci of development in the plant. If two viruses are mutually exclusive, the second hypothesis would suppose the first virus to have " saturated " all the available foci, preventing thereby the multiplication of the other.

SUMMARY

Two strains of the virus of Yellow Mosaic of tomato (Johnson's *T.V. No.* 6) have been isolated and their reactions determined. Evidence is adduced to show that one virus is not an attenuated form of the other, and that many of the anomalies now observed in symptom expression in host plants may probably be referred to the existence of strains not hitherto recognized. It has been found that one strain apparently completely immunizes the host plants against the other. This observation has been extended and it has been found that four types of interaction between viruses can be distinguished.

(*a*) A virus may completely inhibit the development of another in the host tissues.

(*b*) The second virus may multiply in the tissues without inducing typical disease symptoms.

(*c*) The two viruses may multiply and induce symptoms each inducing the typical symptoms of its specific disease.

(*d*) The effect of the second virus may be to cause a more severe disease than either virus could of itself have caused.

The significance of these observations in relation to the multiplication of the virus in the tissues of a host plant is discussed.

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DESCRIPTION OF PLATES

PLATE 14

Fig. 1—Tomato plant infected with virus of the A.Y. strain.
Fig. 2—Similar tomato plant infected with virus of the A.G. strain.

PLATE 15

Fig. 3—Hair of leaf of *S. nodiflorum* showing inclusion bodies typical of *T.V. No.* 6.
Fig. 4—Hair of leaf of *S. nodiflorum* showing inclusion bodies of mixture of *Tom. S.V. No.* 1 and *T.V. No.* 1.
Fig. 5—Leaf of *Zinnia sp.* infected with A.Y. strain.
Fig. 6—Leaf of *N. glauca* infected with A.Y. strain.

Caldwell

Proc. Roy. Soc., B, vol. 117, *Plate* 14
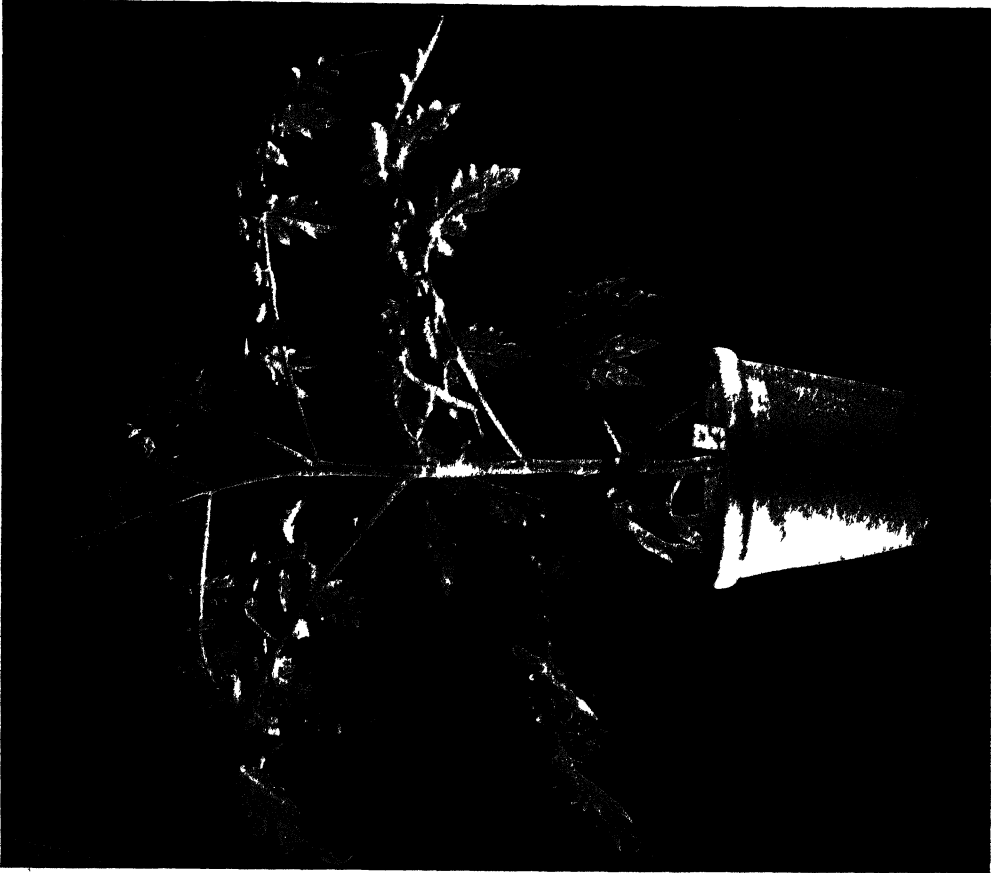
FIG. 2
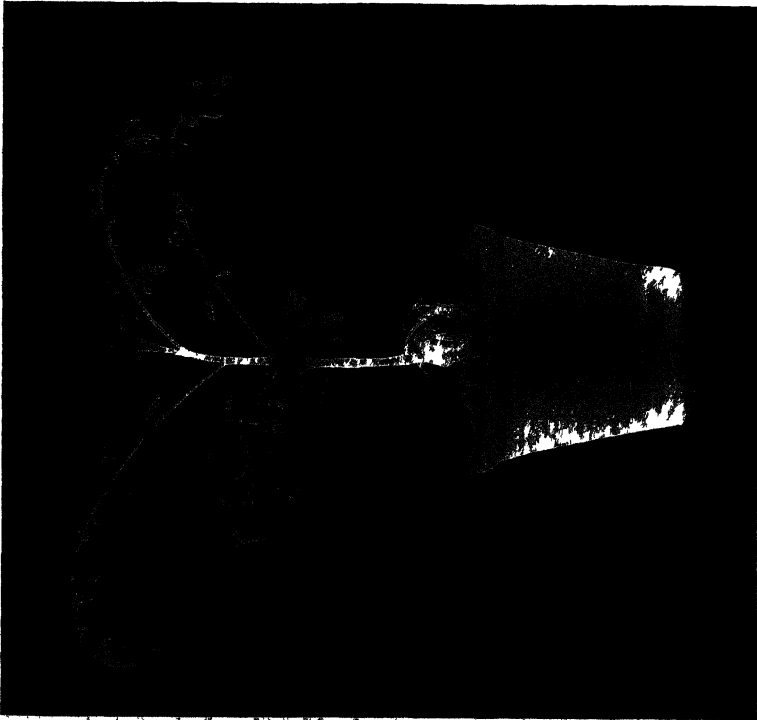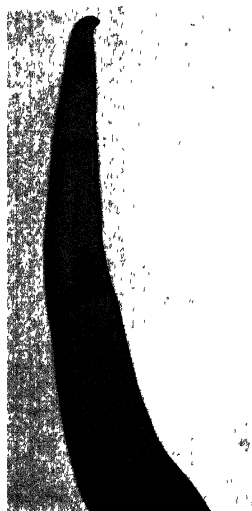
FIG. 1

Fig. 3

Fig. 4

Fig. 5
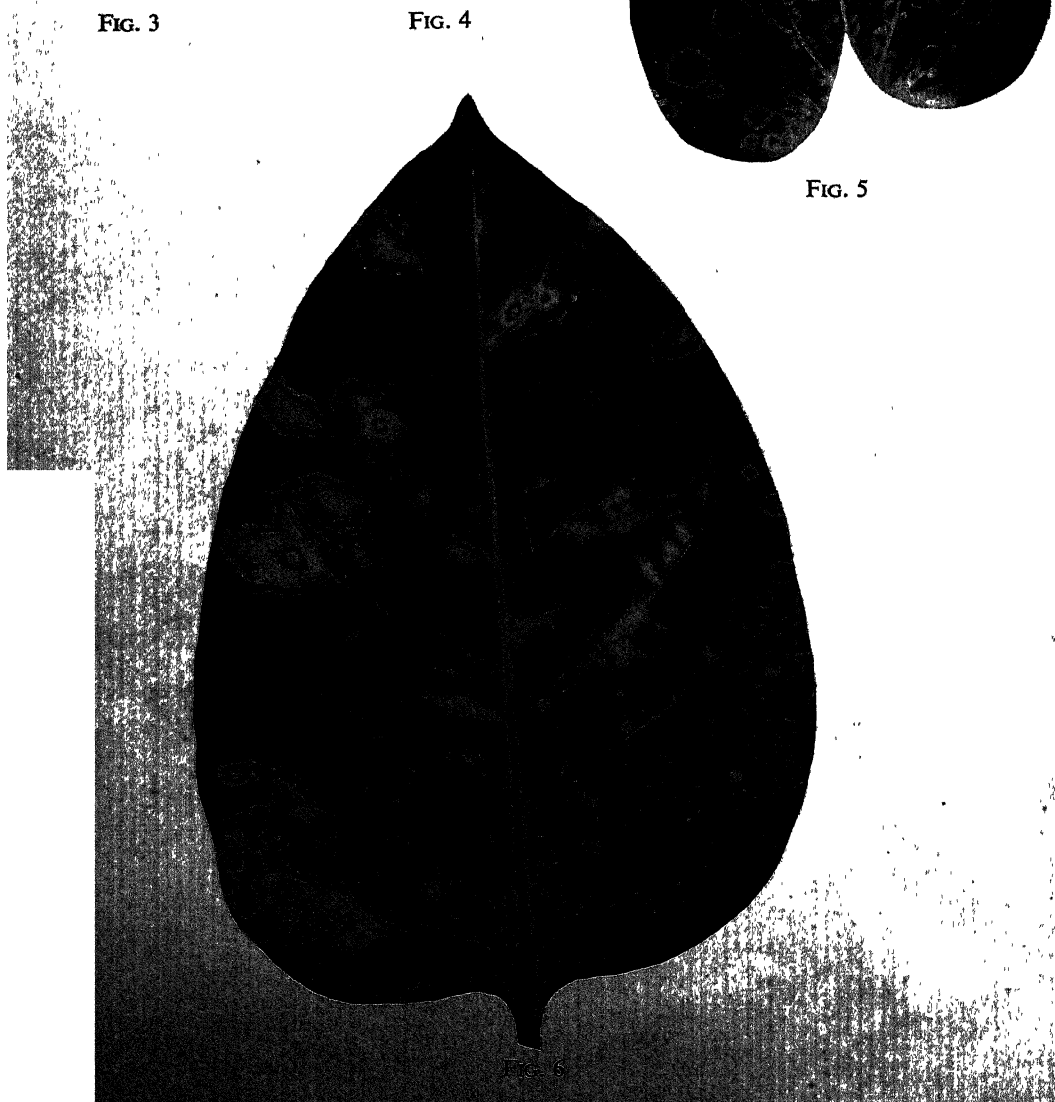
Fig. 6
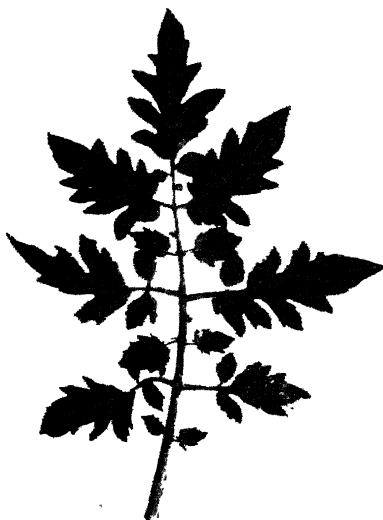
FIG. 7

FIG. 8

FIG. 9

FIG. 10

The Dissolved Constituents of Human Sweat

By A. G. R. WHITEHOUSE, Ph.D., late Tyndall Research Student

(From the Mining Research Laboratory, University of Birmingham)

In this paper the object has been to obtain data as to the dissolved constituents of sweat collected from the human body, and at the same time to endeavour to distinguish what is secreted by the sweat-glands from what is derived from the general surface of the skin.

The experiments were carried out, as previously, in connection with the experimental chamber of the Mining Research Laboratory, University of Birmingham, and mainly during the author's tenure of the Tyndall Studentship. One of the subjects (A. H. H.) was a medical student of good physique who was known to sweat much more freely than the average individual.

METHODS OF ANALYSIS

Most of the methods used in analysing the sweat washings have been described previously (Hancock, Whitehouse, and Haldane, 1929) and it will only be necessary, therefore, to deal with the determinations of lactic acid and urea.

Lactic Acid—The method described by Friedemann, Cotonio, and Shaffer (1927) was employed, using the modified condenser unit of Davenport and Cotonio (1927). The determination consists briefly in oxidizing the lactic acid to acetaldehyde by acid permanganate in presence of manganous sulphate. The acetaldehyde formed is aerated out of solution, absorbed in sodium bisulphite solution, and determined by titration. Immediately after collection the sweat washings were treated for the removal of sugar and other interfering substances by the copper-

lime method of van Slyke (1917). 100 cc of sweat washings were placed in a 200 cc stoppered bottle, 10 cc of 10% copper sulphate solution added, followed by 40 cc of 5% lime suspension. The mixture was shaken at intervals and allowed to stand for some hours, after which the clear liquid was siphoned off or centrifuged. 50 cc of the clear liquid were usually taken for each determination, and after addition of 10 cc of sulphuric acid-manganous sulphate solution, the volume was made up to 90 cc with distilled water. Borax in presence of an excess of glycerol was found to be rather more satisfactory than solid sodium bicarbonate for the liberation of the bound sulphite. Only a small blank (0·20 cc N/100 iodine) was obtained when distilled water was substituted for the sweat washings in the above procedure. Check determinations with zinc lactate were found to give 93% (± 3%) of the theoretical amount of lactic acid.

In this method of determining lactic acid it is very important that a rapid stream of air through the apparatus should be maintained, since low results will be obtained if the aeration is insufficient to prevent decomposition of the acetaldehyde.

Urea—The determination of urea was carried out immediately after each experiment by the direct method of Cole (1931). 50 cc of filtered sweat washings were used, a similar volume being employed for the control. The only differences from the procedure outlined by Cole were that B.D.H. urease tablets were used, and the hydrochloric acid for titration was 0·05 normal.

INORGANIC CONSTITUENTS

Chlorine—The rate of sweating of A. H. H. in Table I was much greater than that found previously with other subjects (Whitehouse, 1931), as was expected, but in addition to this, the chlorine concentration of the sweat was unusually high, ranging from 0·114 to 0·346%. The highest value was found when the subject was at rest in nearly saturated air at 96° F (experiment 49), and sweating was wholly due to the condition of the surrounding air. This value of 0·346% chlorine in sweat approaches very closely to the concentration of chlorine in human blood-plasma (0·36%), but the sweat is still strongly hypotonic, and would need to contain nearly 0·6% of chlorine in order to be isotonic with blood-plasma. Apparent values of from 0·059 to 0·146% chlorine were found with the subject H. W. L. in Table II, but the rate of sweating was comparatively low in most cases. The low chlorine percentages in experiments 54, 55, 56, 58, 59, and 61 are due to the fact that the skin was not

wet with sweat as in the other experiments, and therefore the loss in weight was due partly to osmotic loss of water and partly to sweating, this having been shown in a previous paper (Whitehouse, Hancock, and Haldane, 1932). True values for the concentration of chlorine in the sweat were obtained in experiments 52 and 62 (*b*) where the skin was nearly completely wet, and practically no osmotic loss of water could occur, and it is significant that the loss in weight was also greater in these experiments.

The effect of short bursts of very hard muscular work (7500 foot-pounds per minute) on the chlorine concentration of the sweat was shown in experiments 50 and 51 on A. H. H., when values of 0·292 and 0·276% were obtained. Although the body temperature and pulse rate of the subject in these experiments were both higher than in experiment 49, the percentage of chlorine in the sweat was highest in the latter experiment, in which the subject was at rest. It may be noted that the loss in weight in experiment 51 (*a*) was very nearly 5 lbs per hour, this being remarkably high even when compared with the figure of 6½ lbs per hour found by Moss (1923), for the latter value was obtained from a collier thoroughly acclimatized to working under high air temperature conditions. The rate of work in experiment 60, Table II, was sufficient to nearly double the rate of sweating as compared with experiment 52 in which the subject was at rest, and also caused an increase in the chlorine concentration of the sweat, but the temperature and pulse were raised to higher levels.

It may be concluded, therefore, that the performance of muscular work causes an increase in sweat production, but not necessarily in the concentration of the chlorine in the sweat. If the work performed under certain atmospheric conditions is sufficiently severe, the chlorine concentration is increased but not as much as it is when a similar rise in body temperature occurs with the subject at rest.

Potassium—The concentration of potassium in the sweat of the subject A. H. H., Table I, was found to vary from 0·014 to 0·022%, and is thus similar to that found with previous subjects. The percentage of chlorine calculated as potassium chloride was consistently lower than has been found previously, and did not show the considerable variation during the course of an experiment that was evident with some previous subjects. This is no doubt partly caused by the rate of sweating being high in this series of experiments, for the high values for percentage of chlorine as potassium chloride in previous cases were attributed chiefly to contamination of the sweat by salts present on the surface of the skin. It does seem, however, that the ratio varies with different individuals, and

the variation of the ratio during the course of an experiment also shows characteristic tendencies. The usual proportion of potassium chloride to sodium chloride in sweat during continued or copious sweating appears to be about 1 to 9.

Sulphate—The average concentration of sulphate (SO_4) present as such in the sweat of the subject A. H. H., Table I, was $0 \cdot 004\%$, this usually being about one-fiftieth of the chlorine concentration. The ash sulphate was highest in experiment 45 where the lowest rate of sweating occurred, this being in accord with previous experiments.

The probability of high values for sulphate and ash sulphate in sweat during the initial period of an experiment, particularly when the rate of sweating is low, being due to contamination from the skin, has been referred to previously, and some further experiments were made regarding this matter. In these experiments, the skin of the subject was simply washed down without any previous washing or exposure to high air temperature. In order to keep the volume of washings as small as possible, about half a litre of distilled water was used to wash down a section of the skin surface, the washings being collected in a small foot-bath and employed in turn for washing the remaining sections. After filtration the washings were examined, and the ratio of sulphate (present as such) to chlorine was 1 to 5 in three cases, and 1 to 7 in one case. In a further experiment, the subject was washed down twice in the usual way with a cloth, and the washings rejected. A further wash-down with 2 litres of distilled water was then carried out, using a small rubber sponge to rub the surface of the skin, the washing water being collected in a small foot-bath and used to wash down the skin once more. The washings were filtered, and on analysis gave a figure for ash sulphate which was approximately equal to the amount of chlorine present.

It seems clear, therefore, that the comparatively high ratio of sulphur (either as sulphate or in other forms) to chloride in the initial period of an experiment, particularly when the rate of sweating is low, cannot be regarded as present in the sweat itself, and is apparently due to contamination from the general surface of the skin.

OTHER INORGANIC CONSTITUENTS

Small amounts of calcium and magnesium are apparently present in sweat, as would rather be expected; the amount of calcium as determined by Borchardt (1926) being roughly one-twelfth of the concentration of potassium, while according to Talbert and collaborators (1933) magnesium is present in one-fifth the amount of calcium.

Experiments in which the sweat was tested for the presence of phosphate gave only doubtful traces, and only traces of phosphate have usually been reported by other workers.

<div align="center">ORGANIC CONSTITUENTS</div>

Lactic Acid—Determinations of lactic acid in sweat were made in experiments 52 to 62, considerable amounts being found in every case. Lactic acid has been determined in sweat by several other investigators, and must clearly be regarded as a normal constituent of sweat.

With the subject at rest the concentration of lactic acid in the sweat was about 0·07%, rising to 0·113% in experiment 55, where the surrounding air was at a temperature of 95° F saturated. The normal figure for lactic acid in human blood-plasma is about 0·015%, so that it appears that the sweat-glands are capable of either forming or actively excreting lactic acid to a considerable extent.

In experiments 54 and 58 the subject ran quickly up and down a flight of stairs several times immediately before each experiment, this being, as has often been proved, sufficient to cause a great increase in concentration of lactic acid in the blood. The effect of this severe muscular exertion on the sweat lactic acid was inappreciable. Short bursts of hard muscular work were performed on a bicycle ergometer in experiment 56 and showed what appeared to be an increase in the sweat lactic acid; but in experiment 60, where the conditions of work were much more severe, the concentration of lactic acid in the sweat was identical with the value obtained when the subject was at rest in experiment 52. Experiments 58, 59, and 61 showed that running up stairs before the experiment caused no increase in the percentage of lactic acid in the sweat, and that, quite apart from the performance of muscular work, the proportion of lactic acid to chlorine was high when the rate of sweating was low.

Experiment 62 was conducted in two periods, the sweat being collected separately after each period. It will be seen that the concentration of lactic acid was 0·122% in the first period and 0·089% in the second, while the values for chlorine were 0·085% and 0·117% respectively It appears, therefore, that the proportion of lactic acid to chlorine is particularly high during the initial stage of sweating, as well as when the rate of sweating is low.

In his very interesting book (1934), pp. 231–234, Professor Kuno suggests that the sweat-glands act as excretory organs for the lactic acid discharged into the blood during severe muscular work. The above experiments afford, however, no confirmation of this hypothesis, and

<div align="right">M 2</div>

none of the experiments from this laboratory indicates that the sweat-glands take any active part in regulating the composition of the blood. It appears that we must simply regard the lactic acid in uncontaminated sweat as a normal product of the metabolism of the sweat-glands.

Experiments on simply washing down the surface of the skin, referred to under the section dealing with sulphate in sweat, showed that the material normally present on the skin contained about one and a half times as much lactic acid as chlorine. It seems probable, therefore, that the relatively high lactic acid concentration occurring during the early stage of a test may include lactic acid that has been formed on the skin surface itself, in addition to lactic acid secreted by the sweat-glands.

Urea—Determinations of urea in sweat were made in experiments with the subject H. W. L., in Table II, and the concentration of urea varied from 0·023 to 0·042%. The variation in urea concentration was to some extent parallel to that of lactic acid, the lowest values for urea occurring in experiments 57 and 60 in which the subject performed muscular work.

The concentration of urea in sweat appears to be about the same as in the blood. Gemeinhardt (1933) obtained an average figure of 0·036% of urea in the blood of 1000 non-renal patients, the normal range being from 0·018 to 0·055%. Urea, which diffuses very freely in the animal body, appears to pass into the sweat simply by diffusion. It is true that in cases of Bright's disease of the kidneys the percentage of urea in the sweat may become very high. But the same is true of the percentage in the blood.

Other Organic Constituents

Sugar appears to be a normal constituent of sweat, the amount present being about one-eighth of the urea concentration according to Mosher (1933), and approximately the same ratio was found by Talbert and Silvers (1927).

The data given by Mosher (1933) indicate that about 70% of the non-protein nitrogen of sweat is urea-nitrogen; ammonia-nitrogen and amino-acid-nitrogen amounting to 13% and 4% respectively. The values given for uric acid and creatinine scarcely amount to more than traces.

Organic Matter and Ash

The results given in Table II show that the ratio of organic matter to ash was high in experiments in which the loss in weight through the skin was low, and the proportion of organic matter to ash was lowest (1 to 2·2) in experiment 60 where the greatest loss in weight occurred. Similar

results have been found with previous subjects, and there is thus good evidence that in the early stages of a test, or when little sweating is occurring, sweat is considerably contaminated with the products of broken-down cells from the epidermis and perhaps also the sweat-glands themselves.

The experiments with the subject A. H. H., in Table I, were remarkable for the low ratio of organic matter to ash. In these experiments the sweating was profuse with the chlorine concentration high, and the percentage of organic matter never exceeded one-quarter of the percentage of ash. The progressive decrease in the ratio of organic matter to ash during the course of an experiment was not so evident as with previous subjects, and it is clear that with such a high rate of sweating the effect of contamination from the skin will be considerably reduced. It appears that, as with chlorine, the ratio of organic matter to ash varies to some extent with different individuals.

ASH

If the values for the percentages of chlorine and ash in Tables I and II are compared, there can be no doubt that nearly the whole of the inorganic material in sweat is composed of chloride. With a moderate rate of sweating (about 500 gm per hour) the percentage of chlorine was approximately half the percentage of ash, and this was also found to occur in previous experiments with other subjects. With copious sweating (*e.g.*, experiment 51) the chlorine figure increased to about 54% of the ash, while values as low as 40% only occurred (experiments 56, 58, 59) when the rate of sweating was as low or lower than 400 gm per hour. In a normal case in which the chlorine comprises 50% of the ash, and assuming, as indicated by the experiments, that one-tenth of the chlorine is present as potassium chloride and the remainder as sodium chloride, there would thus be 11% of potassium chloride and 74% of sodium chloride, making a total of 85% of alkali chloride in the ash. Sulphate, assuming it to be present as alkali sulphate, would account for roughly 5% of the ash, so that approximately 90% of the ash would be composed of a mixture of alkali chloride (mainly NaCl) and sulphate. Carbonate, derived from alkali lactate, would practically account for the rest. The actual amount of sulphate present as such in the sweat, however, will be seen from Table I to average about two-fifths of the ash sulphate.

It would seem likely that the lactic acid in sweat is present as sodium or potassium lactate, and that this would be converted during the ashing process into a corresponding amount of carbonate. All the samples of

Experiment No.	Subject and date	Period in hours	Average air temperature in chamber		Wet Kata cooling power	Final mouth temperature °F	Final pulse rate, resting	Total weight lost through skin, gm	Total chlorine, gm
			Dry bulb °F	Wet bulb °F					
44 (a)	A. H. H.	1	108	84	5·2	100·4	115	740	1·900
(b)	23.3.31	1	110	87	4·0	102·2	144	815	2·180
45 (a)	A. H. H.	1	103	88	3·3	99·0	86	450	0·913
(b)	24.3.31	1	107	88	3·6	99·3	91	440	0·774
46 (a)	A. H. H.	1	122	82	6·3	99·1	89	890	1·015
(b)	25.3.31	1	120	82	5·7	99·1	83	700	1·039
(c)		1	123	83	6·5	99·4	94	660	1·192
47 (a)	A. H. H.	1	123	85	4·7	99·5	94	690	1·061
(b)	26.3.31	1	123	90	2·4	100·2	109	820	2·009
(c)		1	123	90·5	2·4	100·8	140	805	2·070
48 (a)	A. H. H.	1	112	93	3·0	99·3	98	1010	1·909
(b)	27.3.31	1	119	92·5	1·8	99·8	110	1220	2·896
(c)		1	121	93·5	2·3	100·4	138	1275	3·116
49 (a)	A. H. H.	1	96·1	95·8	—	99·9	132	1125	3·784
(b)	30.3.31	1	96·3	95·5	—	101·0	154	885	3·063
50 (a)	A. H. H.	1	122	83	5·1	98·9	95	715	0·982
(b)*	31.3.31	1	120	84	5·2	101·6	164	1825	5·325
(c)		1	120	83	5·6	98·8	118	1015	2·676
51 (a)†	A. H. H.	1	120	84	5·5	102·0	158	2240	6·173
(b)	1.4.31	1	123	82·5	5·5	98·7	120	1255	3·039
(c)		2/3	123	82·5	5·2	99·3	130	475	0·874

* Three 10-minute periods of work.

sweat ash that have been examined have given an alkaline reaction, and this has, in fact, been found to be due to the presence of alkali carbonate. In this connection it is significant that in experiments where the ash contained a low percentage of chlorine, experiments 54, 55, 56, 58, 59, and 62 (a), the ratio of lactic acid to chlorine in the sweat was high.

THE REACTION OF SWEAT

The data published regarding the reaction of sweat do not show agreement in general, and sweat has been reported as acid, alkaline, and neutral

Chlorine, %	Potassium, %	$\frac{Cl \text{ as } KCl}{Total \ Cl} \times 100$	Average rate of work, ft-lbs/min	Water drunk during experiment gm	Solid matter, %	Ash, %	Organic matter, %	Total sulphate (SO$_4$) from ash, %	Sulphate (SO$_4$) in collected sweat, %
0·257	0·018	6·3	2560	Nil	0·563	0·513	0·050	0·014	0·004
0·267	0·022	7·6	2865	300	0·567	0·521	0·046	0·008	0·004
0·203	0·021	9·3	Nil	Nil	0·486	0·423	0·063	0·016	0·005
0·176	0·018	9·4	Nil	Nil	0·405	0·375	0·030	0·019	0·004
0·114	0·014	11·0	Nil	Nil	—	—	—	—	—
0·148	0·017	10·5	Nil	500	—	—	—	—	—
0·181	0·018	8·8	Nil	Nil	—	—	—	—	—
0·154	0·018	10·7	Nil	Nil	—	—	—	—	—
0·245	0·019	7·0	Nil	500	—	—	—	—	—
0·257	0·019	6·6	Nil	Nil	—	—	—	—	—
0·189	0·018	8·6	Nil	Nil	0·460	0·375	0·085	0·008	0·004
0·237	0·018	6·7	Nil	500	0·523	0·445	0·078	0·009	0·004
0·244	0·017	6·2	Nil	500	0·556	0·474	0·082	0·010	0·005
0·336	0·017	4·4	Nil	300	0·744	0·654	0·090	0·010	0·004
0·346	0·019	4·8	Nil	300	0·779	0·654	0·125	0·010	0·007
0·137	0·017	10·9	Nil	Nil	0·306	0·254	0·052	0·009	0·001
0·292	0·021	6·4	7540	500	0·659	0·566	0·093	0·008	0·004
0·264	0·017	5·8	Nil	500	0·542	0·500	0·042	0·012	0·002
0·276	0·022	7·3	7720	Nil	0·608	0·506	0·102	0·008	0·004
0·242	0·019	7·2	Nil	1000	0·534	0·467	0·067	0·009	0·004
0·184	0·022	10·8	Nil	500	0·438	0·358	0·080	0·009	0·004

† 43 minutes work in four periods.

by different authors. In the majority of cases, however, the reaction of collected sweat (sweat collected from the whole surface of the body) has been found to be acid, although no satisfactory explanation of the cause of acidity has been advanced. It seemed, therefore, that further examination of this matter was needed.

The sweat was collected from the surface of the skin in a small porcelain crucible, and immediately tested with a suitable indicator. As only a few drops of sweat were available in many of the tests, the most convenient method of determining the p_H value was by means of the " Capillator " (as supplied by the British Drug Houses, Ltd.). The indicators

TABLE

Experiment No.	Subject and date	Period in hours	Average air temperature in chamber		Final mouth temperature ° F	Final pulse rate, resting	Total weight lost through the skin, gm
			Dry bulb ° F	Wet bulb ° F			
52	H. W. L. 28.3.33	1	119	92	99·8	104	505
53	A. G. R. W. 23.6.33	2/3	115	92	99·4	108	530
54*	H. W. L. 20.7.33	1	118	90·5	99·2	98	400
55	H. W. L. 2.8.33	1	95	95	99·7	104	325
56†	H. W. L. 18.9.33	1	93	90	99·5	92	270
57‡	H. W. L. 3.10.33	1	100	87·5	100·1	112	770
58*	H. W. L. 18.10.33	1	117	91	100·1	112	340
59	H. W. L. 15.11.33	1	114	91	100·0	94	295
60§	H. W. L. 6.12.33	1	122	86	101·1	130	910
61	H. W. L. 12.6.34	1	110	93·5	99·8	84	320
62 (a)	H. W. L. 3.7.34	½	110	94	99·8	96	215
(b)		½	115	95	100·2	106	345

* Running up stairs before experiment.
† Two periods of heavy work on ergometer.
‡ Working at rate of 2810 ft-lb/min for 48 minutes.

used were bromocresol green (p_H 3·6 to 5·2), bromocresol purple (p_H 5·2 to 6·8), and phenol red (p_H 6·8 to 8·4), these covering a range of p_H which was sufficient for all the samples examined. A number of the sweat samples were treated with more than one indicator, this being always done when the p_H of the sample was found to be near the limit of the range of the indicator employed. From the results it was clear

II

Total chlorine, gm	Chlorine, %	Solid matter, %	Ash, %	Organic matter, %	Lactic acid, %	Urea %	Sulphate (SO$_4$) from ash, %
0·595	0·118	0·452	0·263	0·189	0·068	—	0·011
1·218	0·230	0·776	0·486	0·290	0·108	0·046	0·013
0·289	0·072	0·335	0·173	0·162	0·079	0·032	0·011
0·263	0·081	0·474	0·191	0·283	0·113	0·042	0·018
0·158	0·059	—	0·156	—	0·106	0·037	0·014
1·011	0·131	0·429	0·276	0·153	0·071	0·023	0·015
0·207	0·061	0·301	0·153	0·148	0·066	0·036	0·010
0·176	0·060	0·258	0·144	0·114	0·074	0·027	0·008
1·325	0·146	0·436	0·300	0·136	0·068	0·026	0·012
0·209	0·065	0·361	—	—	0·097	—	0·010
0·182	0·085	0·454	0·211	0·243	0·122	—	0·011
0·405	0·117	0·434	0·232	0·202	0·089	0·040	0·007

§ At work: 5 minutes at 7140 ft-lb/min, 3 minutes at 8710 ft-lb/min, 10 minutes at 4090 ft-lb/mi₁ 5 minutes at 4090 ft-lb/min.

that sweat has sufficient buffer action for this method of p_H determination.

In the first experiment, the subject (H. W. L.) was seated at rest in the experimental chamber, the air temperature being 115° F dry-bulb and 92° F wet-bulb. The skin had been subjected to the usual preliminary washing that was carried out in all the previous sweating experiments.

Samples of sweat were collected from different places on the surface of the skin at intervals, and the p_H value immediately determined by the method described above. The values obtained are given below:—

	p_H
Leg	4·6
Back (lower portion)	4·8
Back (upper portion)	5·2
Chest	5·2
Face	7·0
Armpits	7·0

Regional variation of the acidity of sweat has been reported by other investigators, e.g., Marchionini (1929), but such low values as p_H 4·6 seem rather remarkable. Professor J. S. Haldane drew attention to the fact that sweat was apparently neutral to litmus when produced on skin that had been freshly washed, and some further experiments were therefore carried out in order to ascertain the effect of more thorough washing of the skin on the leg and back, where the lowest p_H values had been obtained. This " local washing " of the skin was made with a cotton-wool pad and warm distilled water, and followed by drying, but not rubbing, with a clean towel. In order to lessen the interval of time between washing and the production of sufficient sweat for a sample to be collected, the subject worked at a moderate rate on a bicycle ergometer. The surrounding air was kept at a high degree of humidity so as to reduce the rate of evaporation of the sweat.

In the next experiment, in which sweat was collected from the back only, the subject was washed down in the usual way beforehand, and p_H determinations of the sweat were made before and after local washing. The air temperature was 90° F dry-bulb and 88° F wet-bulb.

Local washings were carried out 35 and 65 minutes after the start of the experiment.

Sweat from back (lower)—	p_H
20 minutes after start	4·6
5 minutes after 1st wash	5·8
2 minutes after 1st wash	4·8
5 minutes after 2nd wash	7·2
Sweat from back (middle)—	
20 minutes after start	5·0
5 minutes after 1st wash	7·1
5 minutes after 2nd wash	7·4

Sweat from back (upper)— p_H

    20 minutes after start .... 5·6

    13 minutes after 2nd wash .... 7·4

A further experiment was made in which sweat from the leg (just below knee) was examined. The air temperature was 95° F dry-bulb and 89° F wet-bulb, and local washings were carried out 37, 52, and 63 minutes after the start of the experiment.

Sweat from leg— . p_H

    20 minutes after start .... 4·7

    5 minutes after 1st wash .... 5·9

    7 minutes after 2nd wash .... 6·4

    5 minutes after 3rd wash .... 7·0

    11 minutes after 3rd wash .... 7·1

A sample of sweat from the face collected 5 minutes after local washing was found to have a p_H value of 7·8.

From the above experiments it appears that the highly acid sweat obtained from certain regions of the body owes its acidity to having been in contact with the skin, and further that the surface of the skin in general is strongly acid.

The acidity of the surface of the skin has been measured recently by Schade and Marchionini (1928) with the aid of a specially designed hydrogen electrode. A regional difference in the acidity of the skin was observed, and the values ranged from p_H3 to p_H5. The authors explain the production of the so-called " acid cloak " of the body as a result of sweat becoming more acid during evaporation and impregnating the horny epithelium. Marchionini (1929) infers that the regional difference in the acidity of sweat is due to there being two distinct types of sweat-glands which produce sweat of different hydrogen ion concentration and osmotic pressure. He gives values of p_H4 to 5·5 for sweat from the eccrine glands (scattered over the whole body), while sweat from the apocrine glands (situated chiefly in the armpits) varied from p_H 6·1 to 6·9.

Although the surface of the skin is acid in most places, it does not follow that this is caused by sweating. What is now evident is that sweat itself is normally slightly alkaline like the blood, and that it is not secreted in the acid state in which it is collectable from various areas of the skin. The acid must be formed on the outer surface of the skin, and the fact that the proportions of lactic acid and sulphate were found

to be higher relatively to chloride in the salts present on the epidermis than in normal sweat may be partly accounted for by the presence of uncombined acid. The acid appears to be formed in the epidermis, and even at parts, such as the nails, where no glands of any sort are present; but I have not attempted to follow this matter further.

CONCLUSIONS

1—The concentration of chlorine in the sweat of one subject was usually over 0·15% and in one experiment rose as high as 0·35%, which was much higher than with previous subjects. Severe muscular work raised the chlorine percentage, but not to such an extent as when the same rise in body temperature was produced with the subject at rest, and sweating was wholly due to the condition of the surrounding air.

2—Values for the concentration of potassium ranged in the same subject from 0·014 to 0·022%. The percentage of chlorine calculated as being present as potassium chloride was lower in general with this subject than in the case of previous subjects, the values ranging from 4% to 11%.

3—The concentration of sulphate normally present as such in sweat was found to be low, being approximately 0·004%. High values for the percentage of ash sulphate obtained when the rate of sweating was low, particularly in the initial stage of an experiment, were apparently due to sulphate derived from the epidermal surface.

4—The ratio of organic matter in sweat to ash varied with the duration and intensity of the sweating, and with one subject became as low as 1 to 12.

5—Lactic acid in sweat varied from 0·068 to 0·122%, and since the blood only contains about 0·015% it thus appears that the sweat contains far more lactic acid than the blood. The proportion did not, however, vary with the proportion in the blood, so that the lactic acid must, apparently, be regarded as a normal product of metabolism of the sweat-glands, and not as being actively excreted by them from the blood.

6—The urea content of sweat was found to range from 0·023 to 0·046%, and is thus about the same as in blood-plasma. It seems probable, therefore, that the urea in sweat has simply diffused out from the blood.

7—The reaction of sweat after very careful washing of the skin was found to be slightly alkaline, like that of the blood. Acid sweat (p_H 4·4) was collected from various parts of the body when the skin had not been thoroughly washed. It thus appears that the observed acidity of sweat is communicated to it by the skin surface. The cause of the normal

acidity of the surface of the skin in various parts of the body has not been further investigated, but is quite independent of the reaction of pure sweat itself.

Although this work was chiefly carried out during the author's tenure of the Tyndall Studentship, the main expenses were covered by a grant from the Safety in Mines Research Board, to which the author's acknowledgments are due.

In conclusion, the author wishes to thank Professor J. S. Haldane, F.R.S., for advice and encouragement throughout this work, and also to acknowledge the helpful co-operation of Mr. A. H. Hunt, B.A., and Mr. H. W. Lane in acting as subjects in the experiments.

SUMMARY

The paper contains analyses of the total dissolved constituents of human sweat, including variations in their proportions in different individuals and under different physiological conditions. It was found that in different individuals the proportions of chlorine, sodium, and potassium varied very considerably. Lactic acid was present in pure sweat in much higher proportion than in the blood, but no evidence could be obtained that the sweat-glands take any active part in regulating the proportion of lactic acid or any other substance in the blood. The composition of sweat as actually obtained from the skin without very special precautions was found to be much influenced by substances given off from the general surface of the epidermis; and the ordinary acid reaction of such sweat, which may be as great as $p_H 4 \cdot 4$, was found to be due entirely to these substances, the reaction of sweat itself being slightly alkaline, like the reaction of blood.

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On the Numerical Distribution of Micro-Organisms in the Atmosphere

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1—Introduction

In 1887, Frankland (1887) devised a method for the quantitative estimation of the micro-organisms present in the atmosphere and experimented also (Frankland and Hart 1887) with a method described by Hesse. Frankland published a curve showing the seasonal variation in the number of micro-organisms in the air of London. Saito, in 1904, using the plate method, carried out a series of experiments at Tokyo throughout a whole year with the object of studying seasonal variation. In 1910, Buller and Lowe, using both the plate method and Frankland's quantitative method, investigated the seasonal variation in the number of micro-organisms in the air of Winnipeg. Preliminary observations made by the present author in this country in 1929, showed that the number of micro-organisms falling on plates in a given time vary very greatly with locality. Extreme values were recorded (Horne, 1930, p. 164) for sea-level at Sligachan, Isle of Skye (1 per 100 sq cm per min) and for an orchard at Exning, Cambridgeshire (176 per 100 sq cm per min). It was also observed that the numbers varied with situation in a given locality even when the distance between the centres compared was not very great. It was impossible to discover from the literature on the subject whether any

significance could be attached to the observed differences since none of the earlier investigators had a sound statistical method at their disposal. The work of Fisher (1930) has supplied this need. It was decided therefore to work more systematically in order to discover what principles, if any, govern the distribution of micro-organisms in the air. If the type of distribution could be established, then it should be possible to interpret the experimental results with the aid of methods of statistical analysis.

2—The Experimental Method

The plate method alone was adopted and 10 replicate plates were invariably used for the systematic work. The medium consisted of the following ingredients:—glucose, 2 gm, asparagin 2 gm, magnesium sulphate 0·75 gm, potassium phosphate 1·20 gm, starch 10 gm, and agar 15 gm, dissolved in 1 litre of water. In practice the plates were usually arranged on a stand at a height of 10 feet from ground level and the stand when erected in an orchard was invariably situated in the centre of a group of four fruit trees (Horne (1930), Plates 1 and 2). The length of the exposure was determined by preliminary experiments since, in order to facilitate counting, it was undesirable that the number of organisms falling on any plate should greatly exceed 50. The counts of organisms were made from 4 to 7 days after exposing the plates. The following method of making numerical records proved entirely adequate. The bottom of each plate was marked out into four quadrants. Separate counts were made for the area of medium covering each quadrant, with the aid of a dissecting microscope. When necessary, the quadrants were explored in greater detail with the aid of a microscope. Microscopical exploration proved useful when there was some doubt as to whether a particular growth consisted of fungus or a mixture of fungus and bacteria, also, in distinguishing minute bacterial growths from pellicles of water or of medium. Certain fungi and bacteria grow much faster than others and may spread over slow-growing organisms. The larger growths were carefully examined and it was usually easy to distinguish the included growths owing to some difference in colour, density of mycelium, and so on. The final counts included all organisms which, by means of the methods employed, could be regarded as growing organisms on the occasions when the counts were made. It is, of course, realized that the number of organisms counted will not represent the total organisms coming to rest on the surface of the medium because certain rust fungi, many Basidiomycetes, etc., may remain temporarily or permanently inactive.

The main experimental work* was carried out at the East Malling Research Station and the Horticultural College, Swanley. The plates used for the experiments were almost invariably prepared in this laboratory. After the exposures were made the plates were suitably labelled and returned to the laboratory where all the counts of organisms were made.

At East Malling, plates were periodically exposed in the adjacent plots XIII and XIV situated in the Great East Plantation (East Malling Research Station, pp. 2, 50, 59). These plots differ mainly with respect to manurial treatment, the former having received a balanced ration for some years, whereas the latter has been completely starved (*Ibid.*, p. 198). Each plot consists of both Bramley's Seedling and Worcester Pearmain apple trees grafted on a wide range of stocks. One permanent centre in each plot together with a control centre on open ground were selected for experimental purposes.

At Swanley, plates were exposed periodically at four different centres, three in the orchard and one on arable land as controls. The orchard centres were selected as showing variations in environment and were situated as follows:—W, in section XI of the orchard between Worcester Pearmain and nut trees; B, 80 feet west of W, between Bramley's Seedling trees; BP, 1180 feet west of W, between trees of Bramley's Seedling and Peasgood's Nonsuch.

Additional sets of plates were exposed in the following localities:—

(1) Exning, Cambridgeshire. A large commercial orchard on chalk with trees planted close together with roots below grass. Bramley's Seedling, Cox's Orange Pippin and Worcester Pearmain varieties in separate plots.

(2) Dunadry, Northern Ireland. Isolated well-kept private orchard with about 140 fairly uniform Bramley's Seedling trees widely spaced.

(3) Chelsea Physic Garden. Open situation.

The experimental work was carried out under fair weather conditions with velocity of wind never exceeding 7 miles per hour.

3—Principles Governing the Numerical Distribution of Organisms

Preliminary statistical treatment of the first data obtained suggested the probability of a random distribution of organisms in the air. Fisher

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* The experimental work was mainly carried out by Mr. N. W. Nitimargi, who died in 1931.

(1930) has pointed out, however, that it would be impossible to demonstrate agreement with Poisson series from a single sample of few plates but when a large number of such samples has been obtained under comparable conditions, then for true samples of a Poisson series, the values of X^2, calculated for the samples, will be distributed in a known manner. Fisher gives an example where 100 sets of 6 plates were used for counting bacteria in sugar refinery products and the test of agreement with Poisson series applied. In this experiment, 116 sets, of which 112 sets consisted of 10 plates and 4 sets of 9 plates, were exposed between May 2, 1930, and September 18, 1931, as shown in Tables I–VI. The sets of 9 plates, Table VI, were obviously unsuitable, 7 sets of 10 plates were discarded through accidental contamination, and 5 sets, Table I, May 8, 1931, were omitted in order to leave exactly 100 sets of 10 plates. Apart from these omissions the data have been used without any selection for testing conformity with Poisson distribution by the evaluation of X^2.

Definitely excessive values of X^2 entered in the tables are in bold type. In these cases the average of the plates is not regarded as trustworthy.

TABLE I—EAST MALLING RESEARCH STATION, 1930–31 (5 MINUTES)

Date	Centre	Total	X^2	Fungi	X^2	Bacteria	X^2
1930							
May 2	13 III	46	11·40	9	5·44	37	9·75
,, 2	14 III	137	18·11	14	8·85	123	17·40
,, 29	13 II	123	6·51	45	16·55	78	6·10
., 29	13 III	92	5·82	38	8·31	54	7·89
., 29	14 II	261	15·20	90	15·10	171	10·57
,, 29	14 III	210	13·04	35	7·57	175	10·31
,, 29	O	651	**124·80**	71	5·50	580	**138·27**
June 4	13 I	83	3·63	46	6·61	37	5·43
,, 4	13 II	120	5·83	60	6·33	60	11·33
,, 4	14 I	145	11·52	94	13·66	51	4·49
,, 4	14 III	116	6·24	52	7·61	64	3·18
Oct. 10	13 II	70	2·86	24	4·33	46	1·82
,, 10	13 III	43	6·53	14	7·43	29	5·83
,, 10	14 II	41	8·02	22	4·36	19	11·00
,, 10	14 III	66	8·54	15	3·00	51	13·11
,, 10	O	38	7·79	12	13·00	26	5·54
Nov. 14	13 II	88	3·36	37	12·46	51	2·53
., 14	13 III	60	6·33	14	7·43	46	6·61
,, 14	14 II	95	10·79	39	9·97	56	7·93

13 = Manured plot.   14 = Unmanured plot.   O = Open ground.
I near 10 a.m.   II near mid-day.   III near 5 p.m.

TABLE I—(continued)

Date 1930	Centre	Total	X²	Fungi	X²	Bacteria	X²
Nov. 14	14 III	55	7·73	20	9·00	35	9·86
„ 14	O	132	9·82	23	7·00	109	7·42
Dec. 19	13 II	31	13·19	12	8·00	19	13·10
„ 19	13 III	20	7·00	4	16·00	16	2·75
„ 19	14 II	19	3·63	9	5·44	10	4·00
„ 19	14 III	24	7·66	5	9·00	19	9·95
„ 19	O	36	6·22	13	10·85	23	6·13
1931							
Feb. 20	13 II	26	6·31	3	13·67	23	7·87
„ 20	13 III	24	13·50	10	8·00	14	8·86
„ 20	14 II	35	5·86	7	11·57	28	6·29
„ 20	14 III	16	11·50	9	16·55	7	5·86
„ 20	O	30	9·33	3	7·00	27	8·18
May 8	13 II	32	13·00	23	15·70	9	9·89
„ 8	13 III	45	7·67	9	7·67	36	9·00
„ 8	14 II	15	21·67	8	9·50	7	20·14
„ 8	14 III	42	5·62	9	7·67	33	5·48
„ 8	O	29	19·62	13	21·61	16	**29·00**

13 = Manured plot.   14 = Unmanured plot.   O = Open ground.
II near mid-day.   III near 5 p.m.

TABLE II—EAST MALLING RESEARCH STATION, JULY 24, 1930
(3 MINUTES)

Centre	Total	X²	Fungi	X²	Bacteria	X²
13.1.A	250	8·88	189	8·51	62	·4·45
13.1.B	327	4·04	261	3·25	66	4·60
13.1.C	314	**111·35**	255	**93·43**	59	19·47
13.1.D	333	8·53	270	7·18	63	7·95
13.1.E	287	12·12	215	12·48	72	6·88
13.2.A	274	8·94	238	10·40	36	13·44
13.2.B	438	11·31	375	13·18	63	11·76
13.2.C	391	22·63	343	**23·38**	48	12·42
13.2.D	347	7·90	292	3·82	55	17·54
13.2.E	328	12·57	294	11·78	34	8·94
14.1.A	192	4·77	136	5·91	56	3·28 ·
14.1.B	245	5·08	173	7·87	72	6·61
14.1.C	238	6·45	183	7·98	55	2·27
14.1.D	379	**65·19**	298	**57·17**	81	15·91
14.1.E	254	7·41	194	5·38	60	13·66
14.2.A	314	6·38	248	4·66	66	8·85
14.2.B	331	6·07	273	13·63	58	10·96
14.2.C	286	8·54	239	6·73	47	6·40
14.2.D	311	9·61	258	11·22	53	9·45
14.2.E	325	9·67	259	8·52	66	8·24

TABLE III—HORTICULTURAL COLLEGE, SWANLEY, 1930–31
(5 MINUTES)

Date 1930	Centre	Total	X^2	Fungi	X^2	Bacteria	X^2
May 9	W	369	20·62	103	**23·70**	266	**40·54**
,, 9	B	108	18·11	29	14·79	79	13·03
,, 9	B & P I	57	8·02	31	8·03	26	7·85
,, 9	B & P II	225	· 8·38	95	17·74	130	6·46
,, 16	W	266	9·41	56	15·07	210	7·41
,, 16	B	195	6·79	39	4·33	156	9·51
,, 16	B & P I	213	16·15	44	15·55	169	10·23
,, 16	B & P II	213	5·17·	38	11·48	175	6·43
,, 16	O	187	7·38	45	12·11	142	14·76
June 17	W	1265	**70·95**	530	7·01	735	**90·78**
,, 17	B	269	4·64	178	5·82	91	6·47
,, 17	B & P I	296	**47·90**	113	12·57	183	**54·21**
,, 17	B & P II	411	8·19	157	5·61	254	8·60
,, 17	O	1190	10·35	313	8·17	877	17·31
Sept. 5	W	427	6·70	259	8·84	168	6·52
,, 5	B	267	5·62	167	10·06	100	5·40
,, 5	B P & I	282	3·53	186	10·45	96	10·25
,, 5	O	790	12·30	202	8·10	588	10·26
Oct. 24	W	754	3·48	269	4·19	485	4·26
,, 24	B	477	10·90	211	10·66	266	6·42
,, 24	B & P I	233	3·27	73	8·50	160	5·62
,, 24	B & P II	282	5·23	43	7·46	239	5·64
,, 24	O	506	12·46	297	12·12	209	7·22
Dec. 5	W, G	38	5·68	8	8·50	30	8·00
,, 5	W	38	4·63	11	6·27	27	4·48
,, 5	B	45	16·56	18	14·22	27	11·89
,, 5	B & P I	26	10·15	10	8·00	16	6·50
,, 5	B & P II	49	6·71	29	7·90	20	6·00
,, 5	O	74	5·73	18	9·78	56	5·07
1931 Jan. 22	W	61	9·65	16	16·50	45	3·67
,, 22	B	47	5·55	15	9·67	32	13·00
,, 22	B & P I	51	4·49	15	9·67	36	5·67
,, 22	B & P II	31	11·90	8	4·50	23	11·34
,, 22	O	48	14·08	11	4·45	37	11·91
Mar. 27	W	54	11·92	10	16·00	44	6·45
,, 27	B	48	4·50	2	8·00	46	5·30
,, 27	B & P I	91	3·17	6	7·33	85	2·41
,, 27	O	134	4·06	15	7·00	119	4·11
May 22	W	162	16·02	136	13·71	26	11·70
,, 22	B	39	6·90	32	7·37	7	5·86
,, 22	B & P I	53	2·28	42	2·76	11	10·65
,, 22	B & P II	141	8·85	70	4·85	71	9·70
,, 22	O	46	1·39	32	1·75	14	3·14

W = Worcester Pearmain. B = Bramley's Seedling.
B & P = Bramley's Seedling and Peasgood's I = Near mid-day.
II = Near 5 p.m. O = Open ground. G = Ground level.

A. S. Horne

TABLE IV—EXNING, CAMBRIDGESHIRE, SEPTEMBER 26, 1930
(1 MINUTE)

Centre	Total	X²	Fungi	X²	Bacteria	X²
W	102	15·06	49	14·87	53	9·83
B	132	11·33	61	8·67	71	17·59
C	391	5·55	145	11·34	246	8·00

W = Worcester Pearmain. B = Bramley's Seedling. C = Cox's Orange Pippin.

TABLE V—PHYSIC GARDEN, CHELSEA, JANUARY 9, 1931 (5 MINUTES)

Centre	Total	X²	Fungi	X²	Bacteria	X²
O a	69	5·64	8	2·00	61	5·39
O b	61	7·36	10	6·00	51	7·23
O c	10	10·00	1	9·00	9	12·00

O = Open ground. a = Plates filled with medium. b = Plates partly filled.
c = Plates kept inverted during exposure.

TABLE VI—DUNADRY, NORTHERN IRELAND, SEPTEMBER 17, 1931
(5 MINUTES)

Centre	Total	X²	Fungi	X²	Bacteria	X²
A	282	5·97	198	6·00	84	9·67
B	228	15·56	162	6·89	66	14·71
C	238	12·46	160	7·00	78	10·30
D	219	10·37	158	9·30	61	9·10

The observed frequencies of X^2 for fungi and for bacteria are given in Table VII side by side with those based on theoretical considerations (Fisher, 1930, p. 59, Table VI); there being 10 plates in each set, the values of X^2 found in column 1 were taken from Fisher's Table III for $n = 9$.

The values given in Table VII have been critically examined by Professor R. A. Fisher who considers that the expected and observed frequency distributions of X^2 for the fungi agree fairly well, but with bacteria the agreement is less satisfactory, mainly owing to an excess of high values over 16·919. Nevertheless the correspondence with expectation shown by the majority of the bacterial samples taken in conjunction with the fact that seven of the nine high values of X^2 recorded in Tables I–III and, found apportioned equally among bacteria and fungi, are exceptionally high, suggests that the average of 10 plates is rendered untrustworthy solely through fortuitous circumstances. This view receives confirmation from the full data which show that in seven instances

discrepancy is due to a single plate containing bacterial or fungal numbers far beyond the average, and in only two instances to more than two unsatisfactory plates. The total number of definitely unsatisfactory plates therefore does not greatly exceed 1% of the total used for the entire experiment.

An attempt has been made to determine whether the principles which govern the distribution of fungi or bacteria, considered as a class, will apply when genera or even species are considered separately. No attempt was made to identify the bacteria. The fungi include 38 genera, enumer-

TABLE VII—DISTRIBUTION OF VALUES OF X^2 BASED ON 100 SETS OF 10 PLATES

X^2	Expected	Observed	
		Fungi	Bacteria
0	1	1	1
2·008	1	0	3
2·532	3	2	3
3·325	5	1	3
4·168	10	8	7
5·380	10	9	14
6·393	20	26	24
8·343	20	18	17
10·656	10	10	8
12·242	10	8	7
14·684	5	11	2
16·919	3	1	5
19·679	1	1	1
21·666	1	4	5
Total	100	100	100

ated elsewhere (Horne (1932), p. 280), and these genera differ considerably with respect to frequency of occurrence in plates (*Ibid.*, pp. 281, 282). Whenever practicable, separate counts of genera or species were made. By summing the X^2 values obtained from different experiments in each case, the test of departure from Poisson distribution was carried out. Since in the majority of instances the number of degrees of freedom exceeded 30, the expression $(\sqrt{2x^2} - \sqrt{2n - 1})$ was calculated. If this quantity is numerically greater than ± 2, the value of X^2 is not in accordance with expectation. The results obtained for seven different genera or species are presented in Table VIII giving details for one genus and one species.

As will be seen from Table VIII in no case does the value of $(\sqrt{2x^2} - \sqrt{2n-1})$ exceed ± 2. The values of X^2 are therefore in conformity with Poisson series.

Clearly the results presented in the foregoing pages are capable of giving a valid distribution of organisms, and of each kind, at places and times of exposure.

TABLE VIII—SUMMATION OF VALUES OF X^2

Place	Occasion	No. of sets	S (n)	Total X^2	$(\sqrt{2X^2} - \sqrt{2n-1})$
			Fusicladium		
E.M.	24.7.30	2	16	17·63	+0·37
E.M.	24.7.30	16	144	107·58	−2·27
S.	16.5.30	5	45	47·66	+0·33
S.	17.6.30	5	45	42·87	−0·17
S.	5.9.30	4	36	29·46	−0·76
S.	24.10.30	5	45	40·50	−0·43
E.M.	29.5.30	5	45	54·21	+0·98
E.M.	4.6.30	3	27	27·96	+0·20
	Total	45	403	367·87	−1·25
			Sporotrichum roseum, Link		
S.	9.5.30	5	45	69·22	+2·34
S.	16.5.30	3	27	28·86	+0·32
S.	17.6.30	5	45	31·93	−1·52
S.	5.9.30	4	36	32·45	−0·37
S.	24.10.30	4	36	23·83	−1·53
E.M.	29.5.30	5	45	56·82	+1·23
E.M.	24.7.30	2	18	9·13	−1·65
	Total	28	252	252·24	+0·03
Cladosporium		19	171	144·92	−1·45
Pleospora		11	99	101·31	+0·19
Alternaria..............		8	72	87·23	+1·25
Botrytis		8	72	51·35	−1·82
Epicoccum granulatum ..		10	90	93·24	+0·28

4—NUMERICALLY HETEROGENEOUS POPULATIONS

The data given in Table II may be conveniently used to test whether there is a similar agreement with Poisson series when observations are made simultaneously at different centres within a limited area. In this instance exposures were made at 20 nearly equidistant centres situated within a rectangular area enclosing approximately 5950 square yards. These centres were aligned from north to south in rows of which there

were four, with the first and second situated in plot XIV and the third and fourth in the adjacent plot XIII. Two sets of simultaneous exposures were made, viz., set 1 at 10 centres in rows 1 and 3; and set 2, 20 minutes later and after rain, at 10 centres in rows 2 and 4. In view of the possibility of effects due to time, rain and plot, values of X^2 were calculated for simultaneous sets within each row and the results were combined as shown in Table IX.

TABLE IX—X^2 VALUES. SETS OF PLATES EXPOSED SIMULTANEOUSLY

Plot	Occasion	S (*n*)	Combined organisms		Fungi		Bacteria	
			X^2	P	X^2	P	X^2	P
XIV	1	4	74·61	0	74·71	0	7·88	0·10–0·05
XIV	2	4	3·83	0·50–0·30	2·56	0·70–0·50	4·72	0·50–0·30
XIII	1	4	15·41	0	20·05	0	1·51	0·90–0·80
XIII	2	4	43·69	0	35·88	0	12·94	0·02–0·01
Total......		16	137·54	0	133·20	0	27·05	0·05–0·02

It is clear from Table IX that the results are not consistent with Poisson series, the mixed population is definitely heterogeneous and for this heterogeneity the fungi are almost entirely responsible. The data of fungal and bacterial numbers have been separately subjected to analysis of variance to find out if possible what factors condition heterogeneity. Mean values calculated for all the sets will be found in Table X.

TABLE X—MEAN VALUES. SETS OF PLATES EXPOSED SIMULTANEOUSLY
(EAST MALLING)

	Plot XIV		Plot XIII		Mean
	Occasion 1	Occasion 2	Occasion 1	Occasion 2	
			Bacteria		
	5·6	6·6	6·1	3·6	5·47
	7·2	5·8	6·6	6·3	6·47
	5·5	4·7	5·9	4·8	5·22
	8·1	5·3	6·3	5·5	6·30
	6·0	6·6	7·2	3·4	5·80
Mean	6·48	5·80	6·44	4·72	
			Fungi		
	13·6	24·8	18·9	23·8	20·27
	17·3	27·3	26·1	37·5	27·05
	18·3	23·9	25·5	34·3	25·50
	29·8	25·8	27·0	29·2	27·95
	19·4	25·9	21·5	29·4	24·05
Mean	19·68	25·54	23·80	30·84	

There are in each case 20 sets of 10 plates giving 199 degrees of freedom of which 180 represent error, leaving 1 each for plots (P), occasions (O) and the interaction P and O, and 4 each for centres (C) and the inter-actions, P and C, O and C, and O and P and C. The full results, which for the fungi received corroboration from an analysis made independently by Professor Fisher, are given in Table XI.

TABLE XI—ANALYSIS OF VARIANCE. SETS OF PLATES EXPOSED SIMULTANEOUSLY (EAST MALLING)

	D/F	Σ squares	S.D.	log/$_e$S.D.	Z	1% point
			Bacteria			
Occasion (O) ..	1	72·000	8·48	+2·1383	+1·2208	0·9462
Plot (P)	1	15·130	3·89	+1·3584	+0·4409	0·9462
P and O	1	14·070	3·75	+1·3220	+0·4045	0·9462
Centres (C)	4	45·228	3·36	+1·2128	+0·2953	0·5999
P and C	4	17·240	2·08	+0·7305	−0·1870	1·3000
O and C	4	7·718	1·39	+0·3293	−0·5882	1·3000
O and P and C	4	78·514	4·43	+1·4884	+0·5709	0·5999
Error	180	1127·800	2·50	+0·9175		
			Fungi			
O	1	2080·100	45·61	+3·8199	+1·8970	0·9462
P	1	1109·200	33·30	+3·5055	+1·5826	0·9462
P and O	1	17·400	4·17	+1·4279	−0·4950	4·3794
C	4	1456·960	19·08	+2·9485	+1·0256	0·5999
P and C	4	689·840	13·13	+2·5748	+0·6519	0·5999
O and C	4	756·340	13·75	+2·6211	+0·6982	0·5999
O and P and C	4	216·220	7·35	+1·9950	+0·0721	0·5999
Error	180	8422·700	6·84	+1·9229		

For the bacteria only one result, viz., that given by occasion, is signi-ficant, the value of Z (1·2208) being higher than the value of the requisite 1% point, indicating that the presence of fewer bacteria on the second occasion is not fortuitous. The difference observed no doubt reflects the circumstance that the two sets of exposures were not made simul-taneously but separated by an interval of 20 minutes, during which a shower of rain occurred. With the fungi the differences due to plots, positions within the plots, and times of exposure, are all highly significant as judged by the Z values, which are approximately twice the requisite 1% probability value. The greater density of the fungal population over plot XIII as compared with plot XIV is real, and, in contrast with the bacteria, the number of spores present in the air is increased after rain.

The interactions show positive results, but no insistence on these effects will be made for the following reason. Included among the sets of plates furnishing data for the analysis were three which showed abnormality in distribution; the X^2 values being much higher than would be expected on the basis of Poisson distribution, see Table II, sets 13.1.C, 13.2.C, 14.1.D. The estimation of error therefore is not reliable, and the interaction effects are also suspect. Professor R. A. Fisher, to whom the figures were submitted, recalculated the analysis after adjusting the most aberrant value, thereby eliminating one degree of freedom. The results obtained corroborated the principal results of the analysis in Table XI, the effects of plot and occasion were increased in significance and the effect of position in plot (centres) was slightly decreased in significance but was still highly significant. The interaction effects were not significant.

The data (East Malling) given in Table I may be conveniently used to determine whether effect of plot or of time of day on the density of populations is governed to any extent by the environmental conditions obtaining on particular days. There are six days without rainfall, specified in Table XII, when single sets of 10 plates were exposed on plots XIII and XIV, at midday and again at about 5 p.m., all the sets giving satisfactory values of X^2. The mean values for the requisite sets will be found in Table XII and the analysis of variance in Table XIII.

On comparing the results given in Table XIII with those shown by the first analysis, Table XI, it is seen that the standard deviations due to errors are less than those calculated for the first analysis. The considerable reduction evident in the fungal population is undoubtedly due to using exclusively sets of plates giving satisfactory values of X^2. The first analysis shows that plot is not effective in modifying the density of the bacterial population on a particular day. This result agrees with observations made on five of the six days entering into the second analysis, Table XII. The significant value of Z obtained for plot reflects the exceptional result given on the remaining day, May 29, 1930. With the fungal population, on the other hand, fluctuations in density are more frequently associated with plot as shown by the interactions in which plots and days participate: on certain days the density is higher over plot XIII, on other days it is higher over plot XIV, and the difference in density may be real.

The values of Z calculated for interactions in which the time when observations were made (T) participates, for the bacteria, are highly significant, indicating very real differential effects. The interaction, T and D, reflects the fact that on certain days the density observed at

TABLE XII—MEAN VALUES. SETS OF PLATES EXPOSED ON DIFFERENT DAYS (EAST MALLING)

Day	Time 1 (mid-day)		Time 2 (5 p.m.)		Mean
	Plot XIII	Plot XIV	Plot XIII	Plot XIV	
		Bacteria			
1 (May 29, 1930)	7·8	17·1	5·4	17·5	11·95
2 (Oct. 10, 1930)	4·6	1·9	2·9	5·1	3·62
3 (Nov. 14, 1930)	5·1	5·6	4·6	3·5	4·70
4 (Dec. 19, 1930)	1·9	1·0	1·6	1·9	1·60
5 (Feb. 20, 1931)	2·3	2·8	1·4	0·7	1·80
6 (May 8, 1931)	0·9	0·7	3·6	3·3	2·12
Mean	3·77	4·85	3·25	5·33	
		Fungi			
1	4·5	9·0	3·8	3·5	5·20
2	2·4	2·2	1·4	1·5	1·87
3	3·7	3·9	1·4	2·0	2·75
4	1·2	0·9	0·4	0·5	0·75
5	0·3	0·7	1·0	0·9	0·72
6	2·3	0·8	0·9	0·9	1·25
Mean	2·40	2·92	1·48	1·55	

TABLE XIII—ANALYSIS OF VARIANCE. SETS OF PLATES EXPOSED ON DIFFERENT DAYS (EAST MALLING)

	D/F	Σ squares	S.D.	$\log/_e$ S.D.	Z	1% point
			Bacteria			
T (time of day)	1	0·024	0·15	−1·8971	−2·5902	4·3794
P (plot)........	1	149·784	12·24	+2·5046	+1·8115	0·9462
P and T	1	15·000	3·87	+1·3532	+0·6601	0·9462
D (day)	5	309·749	7·87	+2·0631	+1·8700	0·5522
T and D	5	2908·427	24·12	+3·1830	+2·4899	0·5522
P and D	5	566·667	10·64	+2·3647	+1·6716	0·5522
T and P and D	5	514·149	10·14	+2·3163	+1·6232	0·5522
Error	216	860·600	2·00	+0·6931		
			Fungi			
T	1	79·356	8·91	+2·1872	+1·7298	0·9462
P	1	5·042	2·24	+0·8065	+0·3491	0·9462
P and T	1	2·514	1·59	+0·4613	+0·0039	0·9462
D	5	553·836	10·51	+2·3523	+1·8949	0·5522
T and D	5	105·268	4·59	+1·5239	+1·0665	0·5522
P and D	5	73·982	3·85	+1·3481	+0·8907	0·5522
T and P and D	5	36·278	2·69	+0·9895	+0·5321	0·5522
Error	216	540·400	1·58	+0·4574		

mid-day is greater than, and, on other days, less than the density recorded when exposures were made a few hours later. The value of Z for time is negative and not significant indicating that the opposite effects shown on different days tend to neutralize one another. Time also proved effective in modifying the density of the fungal population; but in contrast with the bacteria, the density tends to be greater at mid-day and is not significantly greater at the later time on any day entering into the analysis.

The work of statistical analysis may be extended further to populations of micro-organisms associated with more diverse environmental conditions than those obtaining at East Malling. For this purpose the experimental results obtained at Swanley, Table III, may be used. The days entering into the intended calculation, specified in Table XIV, are those for which all the sets of plates gave satisfactory values of X^2; and the daily results alone taken into account are those obtained at mid-day in plots W (Worcester Pearmain and Hazel), B (Bramley's Seedling), PB Peasgood's Nonsuch and Bramley's Seedling), and O (open ground). The mean values for bacteria and fungi are given in Table XIV and the analysis of variance in Table XV.

The results given by the analysis, Table XV, undoubtedly reflect the influence of the more varied environmental conditions obtaining at Swanley. The differences of means, for observations made at mid-day, given by the East Malling results (Tables XII, XIII), viz., bacteria, plot XIII and plot XIV, $- 1\cdot08 \pm 0\cdot365$; fungi, plot XIII and plot XIV, $- 0\cdot52 \pm 0\cdot288$, indicate only small differences in density, Whereas, at Swanley the differences of means are as follows:—bacteria, W and B, $+ 5\cdot20$; W and PB, $+ 6\cdot17$; W and O, $- 2\cdot28$; B and PB, $+ 0\cdot87$; B and O, $- 7\cdot85$; PB and O, $- 8\cdot45$, with standard error $\pm 0\cdot537$; fungi, W and B, $+ 7\cdot82$; W and PB, $+ 9\cdot98$; W and O, $+ 4\cdot43$; B and PB, $+ 2\cdot16$; B and O, $- 3\cdot39$; PB and O, $- 5\cdot55$, with standard error $\pm 0\cdot501$. All the differences, with one exception are highly significant, indicating that position where exposures were made proved very effective in modifying the density of the micro-organisms.

The values of Z obtained for days on which observations were made are approximately six times the requisite 1% probability values. Reference to Table XIV will show that this result is mainly conditioned by season. Micro-organisms were much more numerous on summer or autumn days than they were on days during the winter. The East Malling results give much less significant values of Z. The lower significance is mainly conditioned by the observations made on autumn days when, in contrast with Swanley, fewer micro-organisms were

TABLE XIV—MEAN VALUES. SETS OF PLATES EXPOSED IN DIFFERENT POSITIONS (SWANLEY)

Day	Plot				Mean
	W	B	BP	O	
	Bacteria				
1 (May 16, 1930)	21·0	15·6	16·9	14·2	16.92
2 (Sept. 5, 1930)	16·8	10·0	9·6	58·8	23·80
3 (Oct. 24, 1930)	48·5	26·6	16·0	20·9	28·00
4 (Dec. 5, 1930)	2·7	2·7	1·6	5·6	3·15
5 (Jan. 22, 1931)	4·5	3·2	3·6	3·7	3·75
6 (March 27, 1931)........	4·4	4·6	8·5	11·9	7·35
7 (May 22, 1931)	2·6	0·7	1·1	1·4	1·45
Mean	14·36	9·06	8·19	16·64	
	Fungi				
1 (May 16, 1930)	5·6	3·9	4·4	4·5	4·60
2 (June 17, 1930)	53·0	17·8	11·3	31·3	28·35
3 (Sept. 5, 1930)	25·9	16·7	18·6	20·2	20·35
4 (Oct. 24, 1930)	26·9	21·1	7·3	29·7	21·25
5 (Dec. 5, 1930)	1·1	1·8	1·0	1·8	1·42
6 (Jan. 22, 1931)	1·6	1·5	1·5	1·1	1·42
7 (March 27, 1931)........	1·0	0·2	0·6	1·5	0·82
8 (May 22, 1931)	13·6	3·2	4·2	3·2	6·05
Mean	16·09	8·27	6·11	11·66	

TABLE XV—ANALYSIS OF VARIANCE. SETS OF PLATES EXPOSED IN DIFFERENT POSITIONS (SWANLEY)

	D/F	Σ squares	S.D.	$\log/_e$ S.D.	Z	1% point
			Bacteria			
P (plot)........	3	3521·616	10·83	2·3824	1·2255	0·6651
D (day)	6	27947·116	68·25	4·2232	3·0663	0·5152
P and D	18	19920·224	33·27	3·5046	2·3477	<0·3908
Error	252	2547·800	3·18	1·1569		
			Fungi			
P	3	4546·760	38·93	3·6618	2·5081	0·6651
D	7	33775·888	69·46	4·2407	3·0870	<0·5152
P and D	21	9876·545	21·69	3·0769	1·9232	<0·3908
Error	288	2890·100	3·17	1·1537		

recorded. Modifications in the density of the populations associated with change from autumn to winter are responsible to a great extent for the very considerable interaction effects shown by the analysis.

With the Swanley experiments, effect of time of day on the density of the micro-organisms was also given consideration, but the observations were confined to one centre (P B, see Table III). The exposures were made, as before, at times near mid-day and 5 p.m. The days entering into the calculations, with the requisite mean values, are given in Table XVI and the analysis of variance in Table XVII.

TABLE XVI—MEAN VALUES. SETS OF PLATES EXPOSED AT MID-DAY AND 5 P.M. (SWANLEY)

Day	Mid-day		5 p.m.		Mean
	Fungi	Bacteria	Fungi	Bacteria	
May 16, 1930	4·4	16·9	3·8	17·5	10·65
October 24, 1930	7·3	16·0	4·3	23·9	12·87
December 5, 1930	1·0	1·6	2·9	2·0	1·87
January 22, 1931	1·5	3·6	0·8	2·3	2·05
May 22, 1931	4·2	1·1	7·0	7·1	4·85
Mean	3·68	7·84	3·76	10·56	

TABLE XVII—ANALYSIS OF VARIANCE. SETS OF PLATES EXPOSED AT MID-DAY AND 5 P.M. (SWANLEY)

	D/F	Σ squares	S.D.	log/$_e$ S.D.	Z	1% point
Organisms (O)	1	1501·520	38·75	3·6571	2·8199	0·9462
Time (T)	1	98·000	9·90	2·2925	1·4553	0·9462
O and T	1	87·120	9·33	2·2332	1·3960	0·9462
Days (D)	4	4070·020	31·90	3·4626	2·6254	0·5999
D and O	4	2271·910	23·83	3·1710	2·3338	0·5999
D and T	4	178·600	6·68	1·8991	1·0619	0·5999
D and O and T	4	247·222	7·86	2·0618	1·2246	0·5999
Error	180	960·600	2·31	0·8372		

In this experiment the class of micro-organism introduces very real direct and differential effects on the density of the populations. Day is also effective thereby corroborating the results given in Table XVII. The values of Z obtained for time of day and for the interactions in which time participates are highly significant.

5—SIGNIFICANCE OF THE RESULTS

During the course of the experimental work described in the foregoing pages, more than 1000 plates were exposed to contact with the air in

different parts of the country and the fungal and bacterial growths developing therein subsequently counted. As a rule sets of 10 plates were used on any given occasion, and the sets were exposed for 1, 3 or 5 minutes according to circumstances. Values of X^2, calculated separately for the two classes of micro-organisms, were obtained for all the sets and the X^2 values given by 100 consecutive sets of 10 plates were arranged in frequency distributions of fungi and bacteria. In both sets of plates a fairly satisfactory correspondence between the expected and observed frequencies of X^2 values was shown, suggesting conformity with Poisson series. Such discrepancy as occurred between the expected and observed frequencies is mainly due to certain plates, constituting about 1% of the total, where counts revealed an excessive number of micro-organisms, regarded as purely fortuitous, thereby rendering the average of 10 plates untrustworthy. Separate counts were also made for genera or species when circumstances permitted and X^2 evaluated as before. On summing the values of X^2 obtained, in each case, no significant departure from Poisson distribution was observed. It seems reasonable to conclude from the results given by the tests for conformity with Poisson series, that, under fair weather conditions, micro-organisms are distributed at random in the air. Thus, the principles governing the counts of micro-organisms will be exactly the same as those which govern the accuracy of a hæmacytometer count or the accuracy of the technique of dilution, in estimating the numbers of fungal or bacterial growths by dilution method (Fisher, 1930, p. 57). The natural distribution in the air will agree with the distribution in media, where the technique of dilution has afforded a perfectly random distribution of micro-organisms and where these could develop on the plates without mutual interference.

The importance of the recognition that the micro-organisms in the air are distributed in a known manner is at once apparent because, " the idea of an infinite population distributed in a frequency distribution in respect of one or more characters is fundamental to all statistical work " (*Ibid.*, p. 41). It is therefore possible to make valid comparisons of densities of populations of micro-organisms found under various environmental conditions and to estimate the significance of observed differences by the usual methods of statistical analysis. In all comparative work, the use of inefficient statistics is eminently undesirable. Inefficient statistics unavoidably enter into the first analysis, Table XI, alone of those presented in this paper, but in this case it will be noted that by adopting the correction suggested by Professor R. A. Fisher, the significance of the more important results was emphasized and that, simultaneously, the spurious interaction effects disappeared.

The most considerable effects shown by analysis of variance are associated with the day on which observations were made.   The magnitude of the effect is in each case due to including in the analysis days selected from spring, autumn and winter months.   Micro-organisms were least numerous during winter months, then with the advent of spring, the numbers increased, reaching maxima during summer and declining to winter minima during autumn.   The results corroborate those obtained by investigators who had previously studied the effect of season on the numbers of micro-organisms present in the air.   The significant inter-action effects given by the analyses show that day is very often effective in modifying various differential effects associated with position in orchard, time of day, and class of organisms.   The greater magnitude of the interaction effects shown by the Swanley analysis, Table XV, in contrast with East Malling, Table XIII, reflects observations made during autumn; numbers at East Malling declining earlier in the season.   The differences in densities of populations of micro-organisms, associated with position in orchard, recorded for Swanley are very striking.   In contrast with the position between Bramley's Seedling trees or between Bramley's Seedling and Peasgood's Nonsuch trees, micro-organisms were almost invariably more numerous over the position between Worcester Pearmain and Hazel trees, although the distance between positions was not very great.   In contrast with East Malling, the greater hetero-geneity, associated with more varied environmental conditions, of the general population of micro-organisms at Swanley is noteworthy.   Effects associated with time of day vary with the class of organism considered. Thus at East Malling the density of the fungal population observed at mid-day was almost invariably greater than that found a few hours later, irrespective of position in the orchard.   Bacteria, on the other hand, were on some days more numerous, and on other days less numerous at mid-day.   Again the relationship between densities and time of day observed at one position, plot XIII, was not necessarily maintained at the other position, plot XIV, on the days entering into the analysis, as shown by the significant triple interaction effect recorded in Table XIII.   Rain appears to have induced opposite effects on the two classes of organisms, fungi increasing and bacteria decreasing numerically.

Attention has already been directed to the contrast between East Malling, where the orchard is maintained for purposes of research and considerable attention given to cultivation, and Swanley, where cultural conditions vary to a greater extent both as regards degree of hetero-geneity of the populations of micro-organisms and the actual numbers recorded on comparable days.   As far as numbers are concerned the

private orchard at Dunadry, Northern Ireland, which was well-kept, falls between the two, but density varies within very narrow limits with position in the orchard, Table VI. Both wide variation in density with position and very high numbers were found at Exning, where, apparently, the minimum attention had been given to treatment of trees, the numbers estimated for positions between Cox's Orange Pippin trees, Table IV, constituting a record for the entire period covered by the systematic experimental work. It is interesting to note, in passing, that the numbers of micro-organisms recorded for East Malling on occasions in winter agree very well with the number counted, on an occasion in summer, at Sligachan, Isle of Skye, near sea-level, and that approximately twice the number observed at Sligachan were recorded for open situations in London (Hyde Park, and Chelsea Physic Garden) on occasions in winter, bacteria predominating.

Buller and Lowe (1910) suggested that their investigations, carried out on prairie land and in the City of Quebec, might have some bearing on the spread of certain diseases of plants. Attention has indeed been given to the spread of epidemics, notably the spread of wheat rust in the United States and Canada, but the experimental methods used by the earlier workers have not been generally adopted. Instead, various methods of trapping spores have been employed and with considerable success, but such methods are useful solely when the parasite may be easily recognized from spores. In the present investigation, where observations have been principally confined to orchards, the fungi responsible for spotting and decay of apples during storage have been found in the air of orchards from which the stored fruit was obtained (Horne, 1932, p. 285), and in some instances, air infection has been traced to natural hosts (Horne, 1931, p. 277). The plate method, therefore, enables the investigator to hold fungi in culture for the purpose of identification and comparison, and to study relative pathogenicity of growths referred to species or genera. Thus the relationship between the growths obtained via the air, from natural hosts and from cultivated hosts may be ascertained, leading to a clearer understanding of source of infection and consequently facilitating control of fungal diseases of plants.

6—SUMMARY

The plate method, previously used by Buller and Lowe, and others, has been standardized and applied for the purpose of studying the numerical distribution of micro-organisms in the air. Observations were made principally in orchards, selected to show variation in environmental conditions, and at different times of year. As a rule exposures were made on fine days, with wind velocity not exceeding seven miles an hour.

The principal data available consist of numbers of fungal and bacterial growths counted on 100 sets of 10 replicates. The sets or samples were tested for conformity with Poisson distribution by evaluating X^2 and arranging the 100 values obtained for each class of micro-organisms in a frequency distribution (Fisher, 1930, p. 59). The result showed reasonable agreement with distribution expected for true samples of a Poisson series. For fungal genera or species, where fewer samples were available for calculating X^2, the method of summation of values of X^2 (Fisher, 1930, p. 61) was adopted. On evaluating the expression $(\sqrt{2x^2} - \sqrt{2n-1})$ values not exceeding ± 2 were consistently obtained, indicating no significant departure from Poisson distribution. The evidence appears to justify the conclusion that micro-organisms are distributed at random in the air.

The results of statistical analysis, after eliminating inefficient statistics, show that class of micro-organisms, day when observations were made, time of day, position in orchard, and rain, are all effective in modifying the densities of micro-organisms. Real differential effects, associated with class of micro-organisms, day, time of day and position were in many instances obtained.

Observations made in four different localities show differences, associated with locality, in actual numbers of micro-organisms counted, in degree of heterogeneity of populations, and in time of year when numbers decline to the minimum found in winter. These differences appear to be related to the standards of cultivation attained by the orchardists concerned.

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The Embryological Development of
Cheyletus eruditus (a Mite)

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INTRODUCTION

The subject of this research was suggested by Professor J. W. Munro, of the Entomology Department, Imperial College of Science and Technology, to Professor E. W. MacBride, F.R.S., and the investigations were carried out under the supervision of the latter. The mite, on which the present work has been done, belongs to a group of animals of greater economic importance than has hitherto been realized, and hence a knowledge of the early development was thought to be necessary. The embryology of Acárina has never been thoroughly worked out. The only available account of the development of *Cheyletus* is by Kramer (1878), who studied whole mounts only and whose work, therefore, lacks the details necessary to make it complete. The developmental histories of other Acarids, ·e.g., *Tyroglyphus longior, Atax bonzi,* etc., by Claparède (1868) are also incomplete, more attention being paid to the changes in external form than to those of the internal organs, few sections being cut.

The present paper embodies the first account of the formation of germ layers and the development of the various organs of *Cheyletus eruditus,* or indeed in any other mite, starting from the time the egg is laid up to the hatching of the embryo.

The writer takes this opportunity to express his indebtedness and gratitude to Professor E. W. MacBride, F.R.S., for the untiring help and

constant encouragement received from him throughout the course of work. Acknowledgments must also be made to Miss A. M. Hora and ·Mr. C. Horton-Smith for the ungrudging help, they have rendered in facilitating the progress of research.

MATERIAL

The eggs for section cutting were collected by breeding isolated animals supplied by Miss A. M. Hora, of the Entomology Department, Imperial College of Science and Technology. The method for breeding adopted by Newstead and Duvall was followed with certain modifications. Sections of thick glass tubing 2 cm wide and 3 mm deep were cemented on to a glass slide by gold-size and they were then covered with circular coverslips, held in position by rubber bands. The cells were kept in desiccators containing a saturated solution of sodium chloride to ensure the requisite humidity of 75% and the desiccators were placed in an incubator maintained at a constant temperature of 25° C. The animals were supplied daily with the mites, *Tyroglyphus 'longior*, on which they fed, and the cells were examined under binoculars at intervals of an hour for the eggs, which were as a rule laid at night. When eggs were observed in any particular cell, the mother was removed to another cell, to prevent her from devouring them and in order to isolate eggs of similar age. The times and dates were marked on the cells containing the eggs, which were then left in the desiccators until such time as they were ready for fixation. The breeding animal was kept under stricter observation, so that the ages of eggs laid by her could be known with greater accuracy.

TECHNIQUE

Great difficulties were encountered on the technical side of the work and therefore a detailed account of the methods used is given.

The minute size of the eggs (0·15 mm by 0·1 mm) necessitated skilful manipulation in handling them. They were picked up, one at a time, from the cells by the wet point of a very fine needle, taking care not to prick the egg shell. They were then dipped· into a fixative in a solid watch glass, the whole process being carried out with the aid of binoculars. In passing the egg through the successive grades of alcohol, the use of the ordinary pipette was liable to cause the loss of the egg, and fine capillary tubes, of just over 0·1 mm diameter and 10 cm long with one end funnel shaped, were used. By bringing the capillary end of the tube near the egg in the liquid, the capillary action of the tube was used to

draw up the egg with a very small quantity of the reagent. The egg was transferred to the next reagent by dipping the tube into the liquid and by gently blowing through it. Thus the safety of the egg was ensured.

The most satisfactory fixative was found to be Bouin's solution, used at 70° C for 3 hours. The chorion of the egg was fortunately permeable to the action of the various reagents. The egg was stained in light green in absolute alcohol for 20 minutes and then placed on a drop of 8% celloidin solution in a solid watch glass. This was covered by a greased cover glass and left to infiltrate for 2 days. The object sank to the bottom of the celloidin and generally came to rest on one of its sides, so that orientation could be carried out by suitable cutting of the block of hardened celloidin. The position of the egg in the celloidin was more readily determined by placing tiny dots of Indian ink at its extremities. The hardened celloidin was imbedded in wax (56° C) and sections were generally cut at a thickness of 5 μ. Ehrlich's hæmatoxylin and 0·5% eosin in absolute alcohol, as a counter stain, were found to be the most satisfactory stains for the slides.

EARLIER WORK ON ACARIDS

Kramer in his paper of 1876 described the development of the appendages and also the formation of the second egg covering, the " deutovum," and the accompanying apparatus used in breaking up the first egg shell. But he did not describe or illustrate the formation of the internal organs. The five diagrams do not elucidate the points mentioned in his paper. His description of the nymphal stages is less complete than that of Newstead and Duvall (1918), who have determined the time taken for the larva to reach successive ecdyses.

Claparède (1868) has worked out the development of the following Acarids: *Atax bonzi, Myobia, Tetranychus telarius, Tyroglyphus longior,* and *Hoplophora.* It is of interest to note that in *Myobia,* Claparède has described the formation of third egg covering, which he calls the " tritovum," but unfortunately his work does not go beyond the study of whole mounts.

Michael (1895) and Newstead and Duvall (1918) have worked on the anatomy and habits of *Thyas petrophilus* and *Cheyletus eruditus* respectively, but their work deals only with the adult stage. Michael, however, discusses the homology of the special organ of Acarina, viz., the dorsal excretory organ. He could not arrive at definite conclusions since the origin of the organ was not known.

DESCRIPTION

First of all *Cheyletus eruditus* is a parthenogenetic animal. The earliest possible stages of development are observed in the ovary of the brooding adult. Even here, one can only rely on evidence afforded by serial sections, as dissection of the ovary does not reveal all that is necessary for making a thorough study of early development. The serial sections, however, show ova in various stages of development ; the latest stage before oviposition closely resembles the earliest stage after oviposition.

The following is a table of the approximate time taken for the appearance of various organs :—

Blastoderm formation .... 1 to 4 hours
Formation of the middle plate and the lateral
    plates .... 10 to 14 hours
Proliferation of endoderm cells and the over-
    growth of the middle plate .... 20 to 26 hours
Formation of the mesoderm and ectoderm :
    external changes .... 28 to 32 hours
Development of the appendages and the invagina-
    tion of stomodæum .... 36 to 40 hours
Differentiation of nerve cells .... 40 to 42 hours
Completion of the nervous system and the
    establishment of the connection of the stomo-
    dæum to the mid-gut: alimentary system ...... 42 to 43 hours
Beginning of the dorsal excretory canal.......... 43 to 44 hours
Period of delay in the development of the embryo
    and the completion of dorsal excretory canal .. 44 to 62 hours
Beginning of the genital organs and the appearance
    of " blood cells " .... 62 to 66 hours
Appearance of the tracheal system .... 68 to 72 hours
Appearance of the cells of the " salivary glands ".. 80 to 90 hours
Final stage : emergence of the hexapod larva .. 92 to 96 hours

THE ORGANIZATION OF THE EGG AT OVIPOSITION

The egg is 0·15 mm by 0·1 mm broad. It is broadly sub-ovate and the head of the embryo develops at the broad end. The egg has a perfectly smooth surface and when first laid is of uniform opaque white appearance owing to the even distribution of the yolk globules. The chorion is thin and devoid of all sculpture on the surface and there is no micropylar area at either poles. The eggs, when laid, adhere to the substratum by

means of fine threads, probably secreted by the oviducts. Inside the chorion, covering the cortical layer of cytoplasm, is a very fine membrane, which later develops into the second egg shell or the " deutovum."

The cortical layer consists of yolk-free cytoplasm about 10 μ thick. Within it lies the yolk, through which ramify cytoplasmic strands continuous with the cortical layer. The inner surface of the latter has an irregular appearance owing to the cytoplasmic strands.
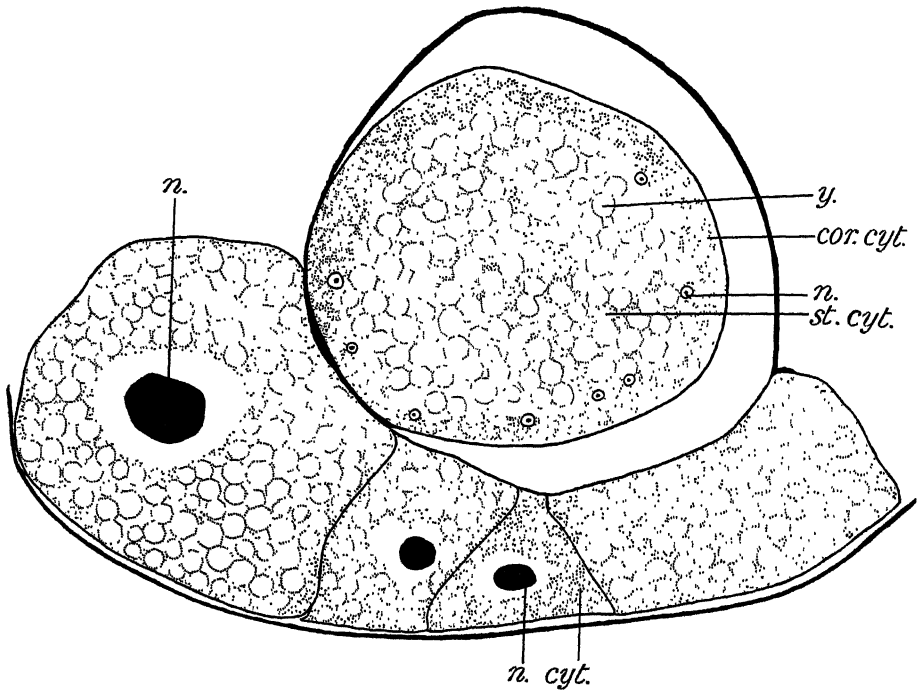
FIG. 1—Transverse section of ovary of a brooding adult showing the ova in various stages of development. *cor.cyt.*, cortical layer of cytoplasm; *n.*, nucleus; *st.cyt.*, streaming cytoplasm ; *y.*, yolk.

All the diagrams were drawn with the aid of camera lucida, using No. 4 eye-piece and 1/12 objective under oil immersion except where otherwise stated

EARLY DEVELOPMENT BEFORE OVIPOSITION

Stage A—As already mentioned, sectioning the brooding adult proved to be the best method for a study of the early development of the egg, fig. 1. In the anterior region of the ovary developing ova are seen in large numbers each consisting of a single large deeply stained nucleus, surrounded by a small quantity of cytoplasm. In a more matured ovum,

the nucleus is separated from the cytoplasm by a clear space. The oviducts contain larger ova with a great number of yolk globules. Subcentrally placed in the egg is a large rounded nucleus, surrounded by the clear space, already referred to in the young ovum.

The eggs occupying the vagina are of different constitution. There is a cortical layer of cytoplasm surrounding the mass of yolk as in a newly deposited egg. There is, however, a little streaming cytoplasm in the meshes of the yolk globules which is in communication with the cortical layer. On the inner side of the cortical layer of cytoplasm, a few nuclei, ranging from four to seven in number, are found. They are, however, considerably smaller in size than the original single large nucleus, found in the earlier ova.

BLASTODERM FORMATION

Stage B (1 minute after deposition)—The nuclei touching the inner edge of the cortical layer have moved a little farther into it and a few of them are found towards its outer edge, fig. 2. The inner streaming cytoplasm still persists, but the inner edge of the cortical layer and the streaming cytoplasm are both observed to be darker in shade than the outer part of the cytoplasm.

Stage C (1 hour)—In an embryo examined 55 minutes after deposition the nuclei are found to have increased in number and have become evenly distributed

FIG. 2—Transverse section of an egg 1 minute after oviposition. Showing the distribution of nuclei in the cortical layer of cytoplasm and the streaming cytoplasm in the meshes of yolk globules is also to be noted. Abbreviations as in previous figure

in the cortical layer, fig. 3. The dark shade of the inner edge of the cortical layer is completely lost and the first indication of the formation of the blastoderm is indicated by the cortical layer of cytoplasm becoming divided into a number of regions each surrounding a nucleus ; that is, definite cells make their appearance. These cells are much less numerous in the posterior than in the anterior region of the ovum.

Stage D (2 hours)—The formation of the blastoderm is complete at about 2 hours after egg laying, fig. 3 (*a*). In this stage, the blastoderm consists of a single layer of cells. The streaming cytoplasm inside has almost disappeared, leaving only the yolk globules, which stain pink with eosin. In the centre of the yolk globules can be seen deep red spots,

probably due to optical illusion.   The cells of the germ layers are stained blue by hæmatoxylin.

It will now be seen that the development of *Cheyletus eruditus* shows the same type formation of blastoderm as that described by Claparède.   In *Tetranychus telarius*, Claparède has shown that the nucleus, surrounded by formative protoplasm, rises to the surface of the yolk and soon divides. Repeated division of the nucleus soon gives rise to a large number of nuclei, each surrounded by an area of protoplasm.   The nuclei remain
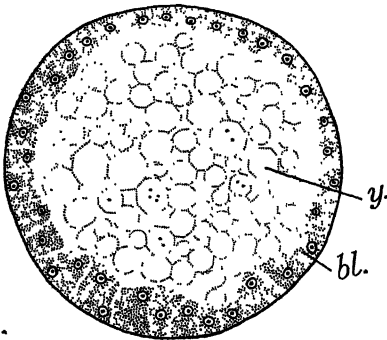
FIG. 3 (*a*)—Enlarged diagram of a portion of the cortical layer of an egg (55 minutes), showing the formation of blastoderm
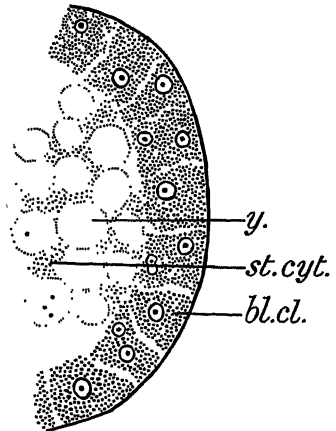
FIG. 3 (*b*)—A small portion under higher magnification. *bl.cl.*, blastoderm cell.   Other abbreviations as in previous figures

lying at the surface of the egg, and by increasing still further in number, they, with the protoplasm around them, give rise to the blastoderm.

THE GERM LAYERS

Stage E (4 hours)—At the time when the germ layers begin to be formed, the blastoderm presents three more or less distinct divisions : (1) a dorsal thin strip, (2) the blastoderm of the lateral surfaces, and (3) the blastoderm of the ventral surface, fig. 5.   Of these three portions, the blastoderm of the ventral and lateral faces of the egg consists of a thick single layered epithelium composed of slender cells.   The thickness is greatest in the anterior region, and decreases slightly and gradually towards the posterior region.   However, at this stage the middle plate is not so distinctly separated from the lateral plates as in a later stage (13 hours).

PROLIFERATION OF ENDODERM CELLS

Stage F (14 hours)—From the middle plate a few comparatively large cells become differentiated and are seen to have shifted towards the yolk mass, fig. 4. These cells, many of which in the section figured are actually engaged in dividing, later migrate inwards into the yolk mass and thus represent the endoderm. A similar state of affairs has been observed in the Araneæ.
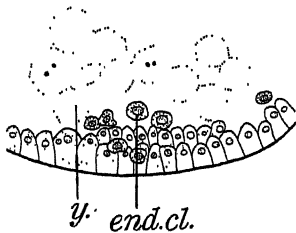
FIG. 4—Section of an egg (13¾ hours), enlarged, showing the proliferation of endoderm cells from the ventral portion of the germ band.   *end.cl.,* endoderm cell
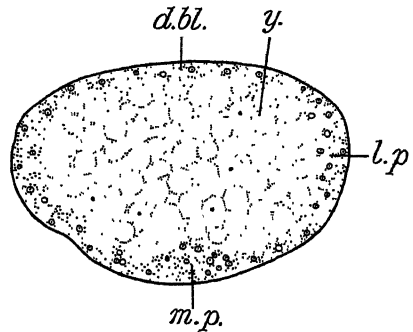
FIG. 5—Transverse section of an egg about 26 hours in development, showing the thickenings of the lateral plates and the middle plate. *d.bl.,* dorsal portion of blastoderm; *l.p.,* lateral plate; *m.p.,* middle plate

According to Kowalevsky (1887), Schulgin (1887), Laurie (1890), and Brauer (1894), the inner surface of the germ disc of a scorpion's egg is quite irregular, for single cells become detached from it which shift into the yolk and these cells give rise to the " yolk cells," which bring about the disintegration of the yolk, without, however, taking part in the formation of the endoderm of the embryo.

But in Araneæ, according to Morin (1887), thickening of the blastoderm arises in the region which corresponds to the later ventral surface, *i.e.,* the rudiment of the germ band; not only do the cells here increase in size, but some of them separate from the blastoderm and form definite layers. At the same time a few cells in this region become entirely disconnected from the rest and migrate into the yolk, representing the endoderm. These Morin calls the " yolk cells."

In the eggs of the Araneæ, besides the single nucleus, a remarkable structure is found in them called the " yolk nucleus "; but this is not yet sufficiently understood. It consists of a compact accumulation of spherules; occasionally it is quite a complicated structure, composed

of several concentric layers. When the egg matures the yolk-nucleus usually disappears. A yolk-nucleus of this type is not observed in the egg of *Cheyletus eruditus*. Nevertheless, it is obvious that the endoderm cells, proliferated from the ventral portion, not only take part in forming the endoderm layer covering the mid-gut, but also act as the disintegrating agents of the yolk mass.

Stage G (26 hours)—In the further development of the germ band the middle plate is overgrown by the downward growth of the lateral plates and is pushed up into the yolk. The middle plate in this stage appears as a longitudinal protruberance along the mid-ventral line, fig. 6, more defined in the caudal region than in the head region. The middle plate,
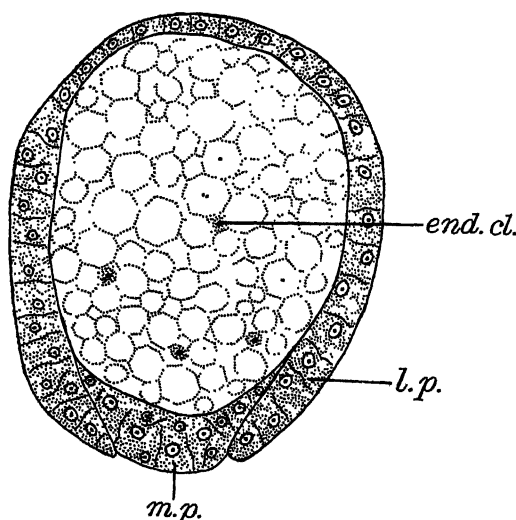
FIG. 6—Transverse section through the anterior end of the egg showing the overgrowth of the middle plate. Abbreviations as in previous figure

which constitutes the future mesoderm, however, remains undifferentiated from the lateral plate in the anterior region, the germ band in this region being of relatively enormous thickness and having the appearance of a recurved hook. A little behind the anterior tip, the germ band shows a great thickening in the mid-ventral region extending to the dorsal surface, thereby leaving on either side two pockets of yolk, fig. 9. These are the rudiments of the first pair of diverticula of the mid-gut of the embryo. In a sagittal section, the ventral plate appears to bend dorsally at a point one-third of the distance from the tip of the head lobe to the opposite extremity.

At the same time the endoderm cells budded off from the middle plate become detached from it and are found scattered among the yolk globules.

They have considerably increased in number, especially in the posterior region which is destined to be the future mid-gut of the embryo, fig. 7.

Stage H (32 hours)—The overgrowth of the middle plate is marked in this stage. The lateral plates have increased in thickness and have grown downwards towards the mid-ventral region. The middle plate
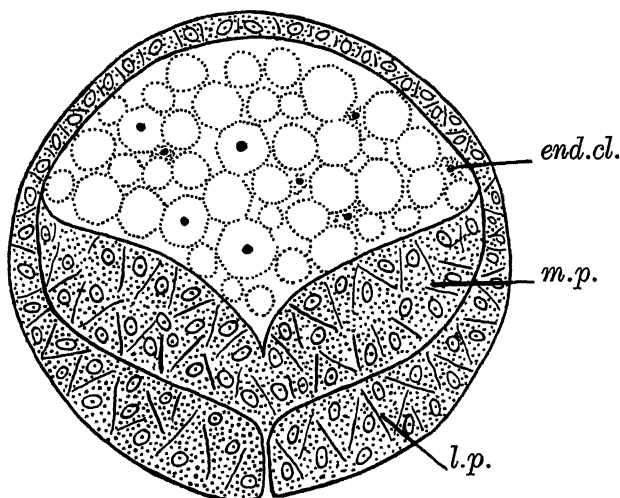
FIG. 7—Transverse section of a more advanced egg through the middle region showing the further overgrowth of the middle plate. The endoderm cells are occupying a more peripheral position. Abbreviations as in previous figures.

lies supported partly on the two lower ends of the lateral plates, fig. 7. The dorsal continuation of the lateral plates remains very thin. The endoderm cells after leaving the central portion of the germ band have now reached a more peripheral position.

Stage I (35 hours)—By their downward growth the lateral plates have united in the mid-ventral line and the middle plate has come to lie against the inner side, fig. 8. The formation of the ectoderm (*l.p.*) and the mesoderm (*m.p.*) layers is now complete. The endoderm cells arrange themselves in contact with the mesoderm layer on its inner side thereby giving rise to the endoderm layer. In a transverse section, the middle plate shows great thickness, and has become flattened by the increase in thickness of the two fused lateral plates.

The overgrowth of the middle plate by the lateral plates may be regarded as an invagination of the middle plate into the interior of the egg, such as occurs in the egg of *Astacus*. The middle plate as in *Astacus* also gives rise to the cells which go into the yolk and form a covering, and thus eventually form the definitive epithelium of the mid-gut.

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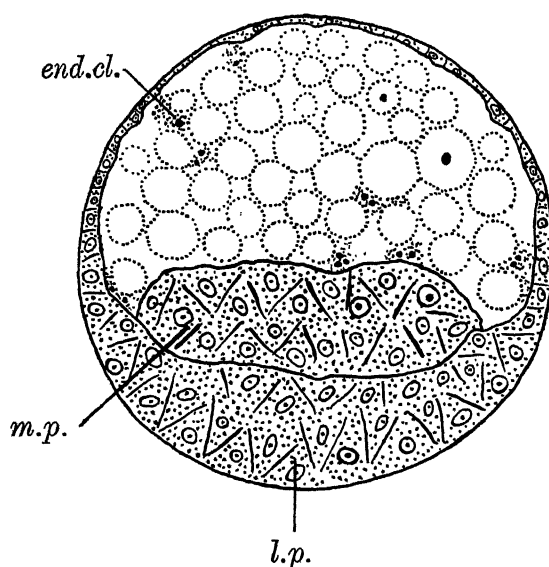
Fig. 8—Transverse section of a still more matured egg showing the completion of the formation of germ layers. Abbreviations as in previous figures
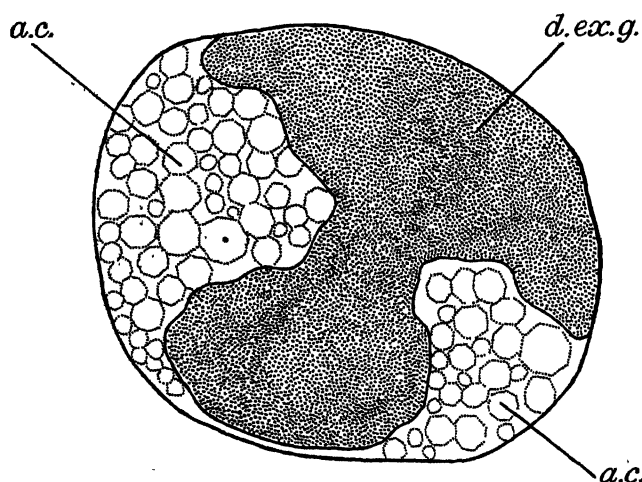
Fig. 9—Transverse section through the anterior region of the embryo, to show the enormous thickening of the germ band in the head region. *a.c.*, rudiments of the anterior cæca of the gut; *d.ex.g.*, dorsal extension of the germ band

DEVELOPMENT OF NERVOUS SYSTEM AND THE STOMODÆUM

Stage J (37½ hours)—The stomodæum arises as an ectodermal invagination in the region of the head lobe near the dorsal surface, fig. 10. The germ band immediately below the stomodæum later gives rise to the rudiments of the appendages of the head. The invagination increases in length, at the same time becoming narrower.

On the ventral side of the head lobe, a group of ectodermal cells become differentiated (*n.g.*, fig. 12). These cells, which give rise to the nervous system, show their different characteristics by their failure to
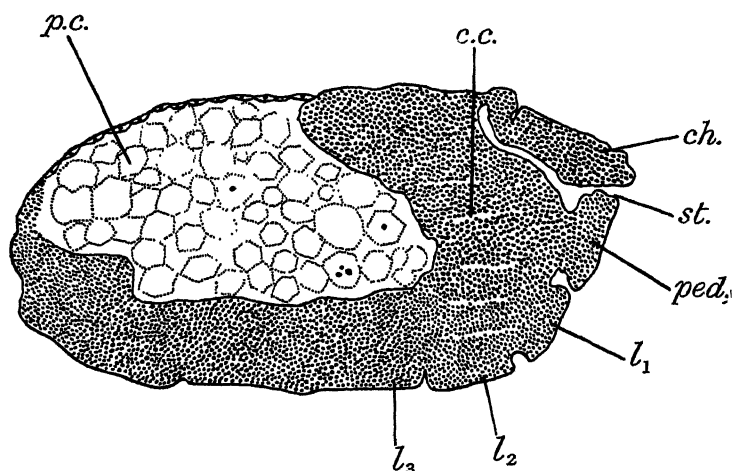
FIG. 10—Sagittal section of the embryo to show the ectodermal invagination of the stomodæum, the dorsal extension of the germ band and the rudiments of appendages. *c.c.*, coelomic sacs; *ch.*, rudiment of cheliceræ; l_1, l_2 and l_3, rudiments of the first three legs on one side; *p.c.*, rudiment of posterior cæcum of the gut; *ped.*, rudiment of pedipalpi; *st.*, rudiment of the stomodæum

absorb either eosin or hæmatoxylin stains. The cells thus appear as a pale mass lying in the ectoderm of the germ band.

EXTERNAL CHARACTERS

The enormous growth of the germ band in the anterior end of the embryo indicates the region of the future rostrum. The germ band below and behind the stomodæum shows five pairs of thickenings, which represent the rudiments of the appendages, figs. 10 and 11. The first of these is very small, representing the pair of cheliceræ and occupying the tip of the embryo. The second pair, corresponding to the pedipalpi, occupies a position immediately behind the cheliceral rudiments. The

rudiments of the pedipalpi are, however, slightly separated from each other, so as to accommodate the rudiments of the chelicerae in the space between them. The last three, more prominent pairs of thickenings, occupy the ventral region of the embryo extending to about a third of the distance from the posterior end. They represent the three larval legs of the embryo. Each of these is separated a little from its fellow.
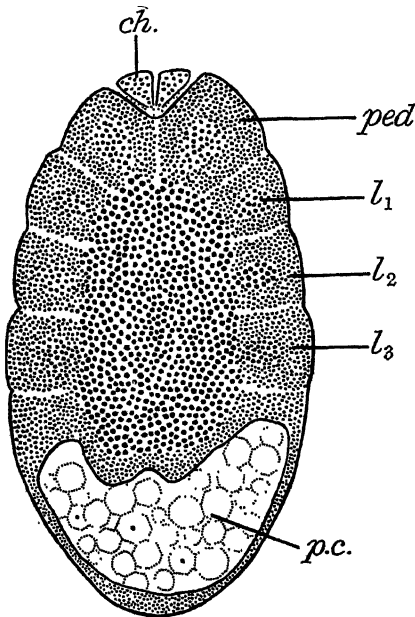
FIG. 11—Frontal section of an embryo running slightly above the ventral aspect, showing the rudiments of chelicerae, pedipalpi, and the three larval legs. Abbreviations as in the previous figure
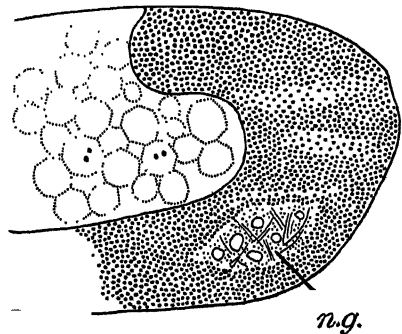
FIG. 12—Sagittal section of the embryo through the anterior region to show the differentiation of a mass of lightly stained nerve cells from the ectoderm on the ventral side of the germ band. *n.g.*, rudiment of the nerve ganglion

Stage K (41½ hours)—The nerve mass migrates upwards and occupies the centre of the huge head lobe, fig. 13. The stomodæum at the same time grows rapidly backwards, and forms the œsophagus piercing the single huge ganglion. It then runs dorso-ventrally and opens into the spacious yolk cavity at the posterior end. The lumen of the œsophagus being very minute, sagittal sections did not show this so clearly as did the transverse sections.

At this stage the single ganglion has developed on its antero-ventral surface five paired lobes, the whole ganglion being surrounded by a thick cortical layer of cells. In an earlier stage, the five paired lobes do not all
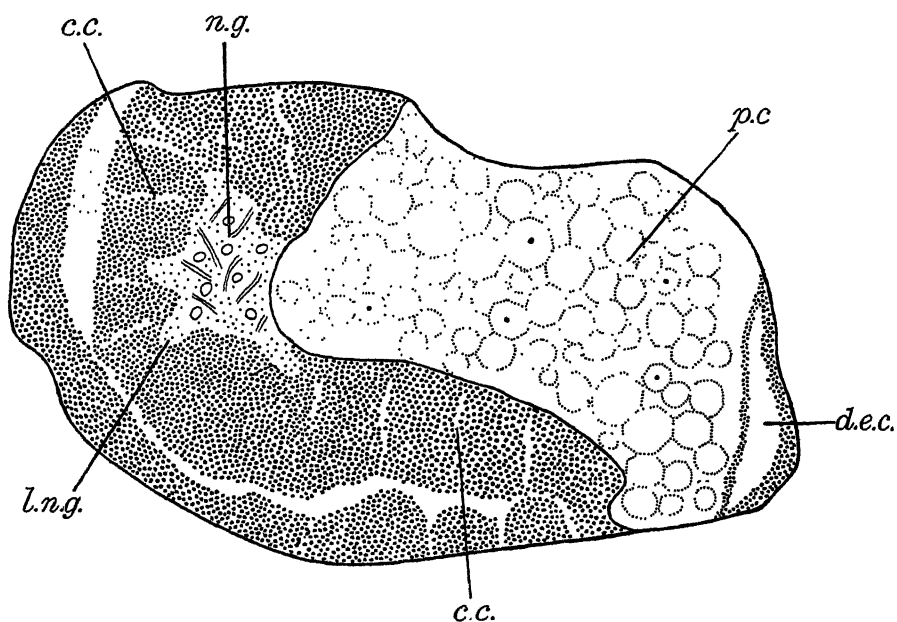
FIG. 13—Sagittal section of a more advanced embryo to one side, showing the position of the nerve ganglion in the head lobe, early segmentation, and the posterior end of the developing dorsal excretory canal. *d.e.c.*, rudiment of the dorsal excretory canal; *l.n.g.*, lobe of nerve ganglion. Other abbreviations as before
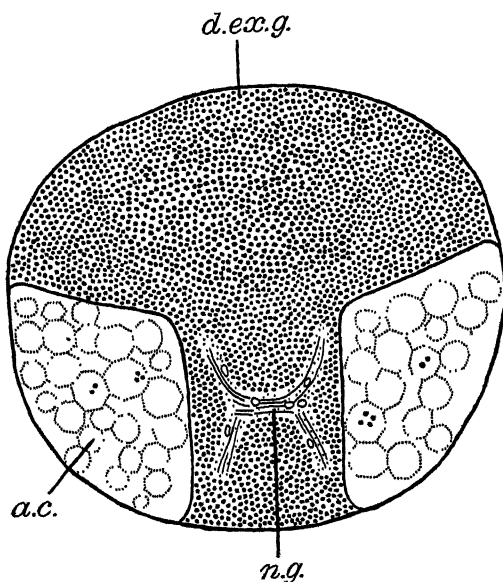

FIG. 14—Transverse section of the embryo about the same age as the previous one, showing the nerve mass and the pair of anterior cæca. *a.c.*, anterior cæca of the yolk mass ; *d.ex.g.*, dorsal extension of the yolk mass

point downwards, but the most anterior ones are turned antero-dorsally. This appears to correspond to the then position of the most anterior appendages, the rudiments of the chelicerae and pedipalpi which occupy a slightly antero-dorsal position. Further, there is definite indication of a segmentation of the mesoderm, which shows itself in sagittal sections. On the ventral side, corresponding to each pair of lobes of the nerve ganglion is a splitting of the mesoderm representing a cœlomic sac and farther backwards in the region of the future abdomen are four such spaces. These spaces are the only evidence of metameric segmentation of the body, which in later stages completely disappears, for no such segmentation can be seen in the adult anatomy. The five paired lobes of the nerve mass may represent the ganglia, which correspond to the five larval paired appendages and which are in the very early stage fused into a single ganglion.

Stage L (43 hours)—The yolk sac now assumes the nature of mid-gut, its surface having become covered with a layer of endoderm cells which have migrated outwards. The sac, however, has become divided into three parts, a posterior spacious cæcum and a pair of anterior pockets running forwards on either side of the nerve ganglion.

The larval appendages still retain their blunt sausage-like shape, but are bent inwards, so that the distal ends of each member of a pair are almost touching.

DEVELOPMENT OF THE DORSAL EXCRETORY CANAL

Stage M (44 hours)—This important organ is initiated a third of the distance from the anterior end, along the mid-dorsal line by a slight intucking of the ectoderm. This intucking though slight in the anterior end is very much pronounced at the posterior end, where it is continued downwards to the extreme end of the ventral side. By the deep inpushing of the ectoderm on the extreme posterior end in this region, the yolk thus becomes divided into two blind pockets, which ultimately form the posterior pair of cæca of the adult animal.

The ectodermal intucking on the dorsal aspect of the embryo then deepens considerably and assumes the form of an open longitudinal groove, fig. 15, bending sharply downwards at the posterior end, fig. 15 (*a*). Its sides by this time have come together at the hind end thus forming a tube posteriorly, which opens to the exterior on the ventral side. The aperture, however, takes the shape of a longitudinal split, a condition similar to that in the adult.

The dorsal excretory canal is then completed by the groove becoming closed from behind forwards, with the anterior end of the organ ending blindly, fig. 19. Intermediate stages, however, show the posterior part of the dorsal excretory canal closed and the anterior dorsal portion in the form of an open groove. The permanent opening of the dorsal excretory canal, however, is at its extreme posterior end, *o.d.e.c.*, fig. 19.
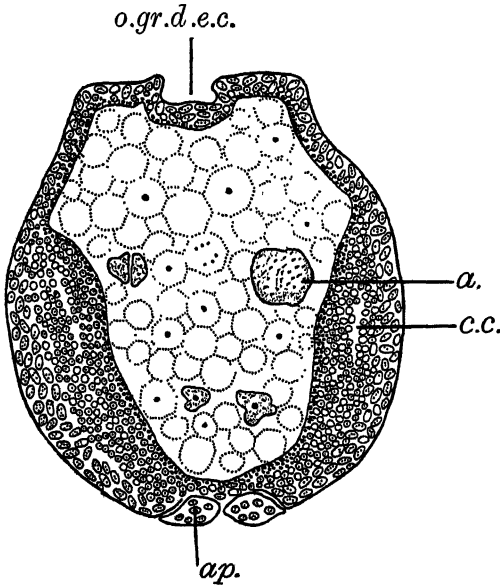

FIG. 15—Transverse section through the abdominal region of the embryo to show the open groove of the dorsal excretory canal, the presence of large amœboid shaped "blood cells" floating in the body cavity and the narrow coelomic cavities in the mesodermic blocks on either side. *a.*, large amœbocyte in the yolk; *ap.*, appendage; *c.c.*, coelomic sac; *o.gr.d.e.c.*, opening of excretory canal
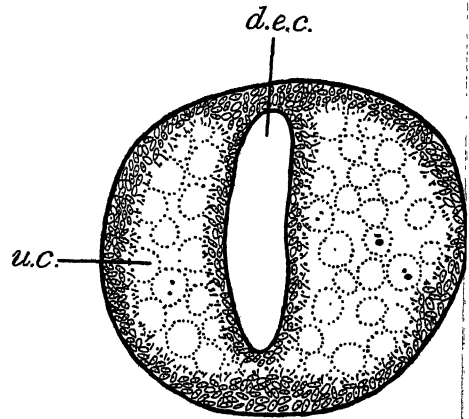
FIG. 15 (*a*)—Penultimate transverse section the embryo showing the posterior part the developing dorsal excretory canal a the division of the posterior cæcum int pair of blind diverticulæ. *d.e.c.*, do excretory canal; *u.c.*, posterior cæcum

It is thus seen that the origin of this much-discussed organ is purely ectodermal. At no time throughout the course of development is any communication found between this organ and the mid-gut, which in *Cheyletus* is developed out of the yolk sac. The absence of an anus in *Cheyletus eruditus* and allied species with similar anatomical features must now be regarded as proven. However, Schaub (1889) described two separate openings in the anatomy of a *Hydrachnid of*

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Pontarachna one of these he regarded as the true anus. With this single exception, there is not on record one species which possesses an opening of the dorsal excretory canal together with a true anus. Even Michael (1895), who studied the anatomy of *Thyas petrophilus* in great detail, failed to see the second opening. In his paper on this animal he attempts to homologize the dorsal excretory canal with one of the Malpighian tubules of other Arachnida. But he is inclined to believe that the organ is more likely to be homologous with the hind-gut than with the Malpighian tubule, and the present writer prefers to agree with him, especially since, so far as is known at present, the origin of the Malpighian tubule in Arachnida is endodermal. Wagner (1894) has proved that in the embryo of *Ixodes* the Malpighian tubules are definitely endodermal in origin. It is now suggested that this organ, which is the early proctodæum of the animal, fails to establish a connection with the mid-gut and assumes the nature and function of an excretory organ.

FURTHER DEVELOPMENT OF EXTERNAL CHARACTERS

Stage N (62 hours)—By about 62 hours the dorsal excretory canal has attained its full development and the limbs of the embryo have acquired articulations, fig. 16, so as to possess three joints, the distal joint of each limb being approximated towards the mid-ventral region, while the proximal joint is continuous with the lateral portion of the germ band. The cheliceræ and the pedipalpi are now bent down with the large ventrally curved rostrum, fig. 21.

DEVELOPMENT OF THE GENITAL ORGANS

Stage O (66 hours)—Just about the region of the third pair of larval legs, the mesoderm band shows on its lower edge certain specialized cells fig. 17. These cells, which are the beginning of the ovary of the adult, thus arise as a paired group of mesoderm cells in the posterior portion of the body of the embryo. They are, however, later found to fuse into a single ovary in the mid-ventral region. These genital cells lie more or less on the walls of the abdomen.

BLOOD VASCULAR SYSTEM : " AMŒBOCYTES "

At this stage the " blood cells " are found to arise. Immediately above the genital cells, from the splanchnic layer of mesoderm blocks, fig. 17, can be seen one or two large amœboid shaped cells with ill-defined nuclei
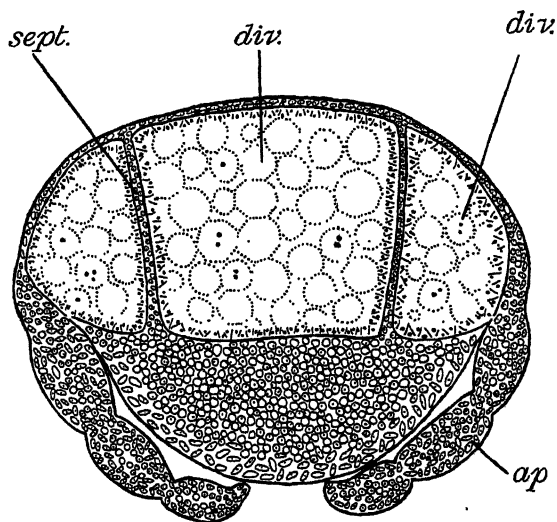
FIG. 16—Transverse section passing immediately behind the region of the brain. This shows the thick ventral portion of the germ band, the development of a pair of mesodermic septa in the cæcum, and the joints of the first pair of larval legs. *ap.*, appendage; *div.*, diverticulum of the gut; *sept.*, septum
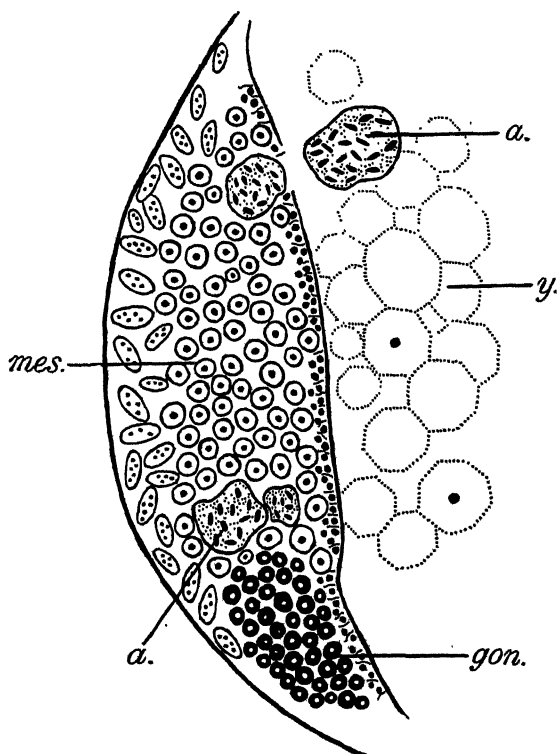

FIG. 17—An enlarged diagram of the embryo in the posterior region showing the development of the gonadial cells and the origin of the " blood cells." *a.*, amœbocyte; *gon.*, rudiment of the gonad; *mes.*, mesoderm; *y.*, yolk

in the process of migrating into the mid-gut, fig. 15. A few such " amœbocytes " about double the size of the largest yolk globules are found floating in the spaces ramifying in the mass of the mesoderm. These cells, however, persist till a very late stage, after which they appear
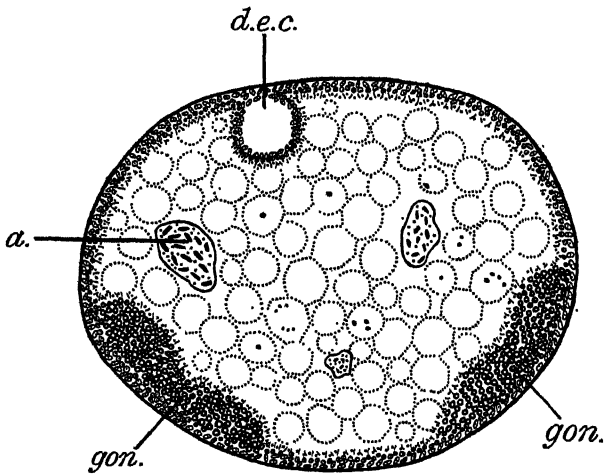
FIG. 18
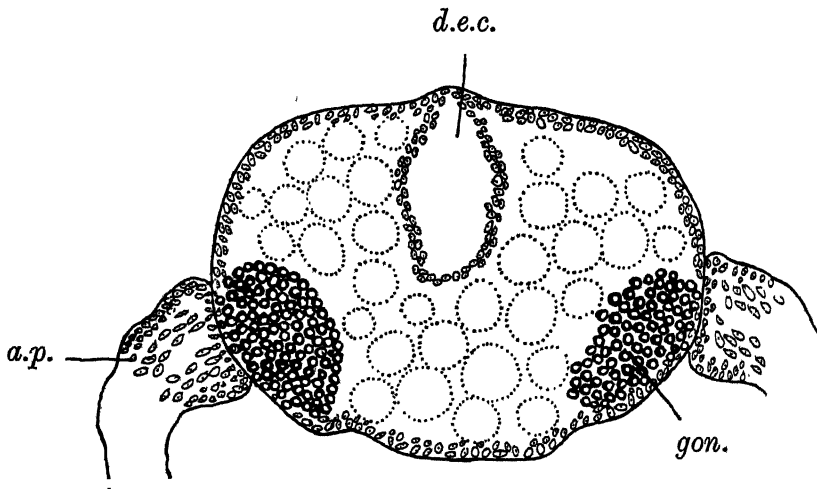
FIG. 18 (a)

FIGS. 18, 18 (a)—Two sections through the hinder portion of an embryo showing the later development of the gonadial tissue. *d.e.c.*, dorsal excretory canal

to have been absorbed. In the adult the writer could not discern such " blood cells," and Newstead and Duvall's (1918) anatomy of *Cheyletus eruditus* does not mention any blood-vascular system at all.

DIVISION OF THE MID-GUT

Behind the nerve ganglion, the median block of cells of mesoderm gives rise to a pair of longitudinal septa on either side growing out in the region of the second pair of legs, fig. 16. In this way the main portion of the mid-gut becomes divided into a pair of diverticula. As already explained, the posterior end of the mid-gut is constricted by the posterior end of the dorsal excretory canal and the anterior end by the nerve ganglion forming two pockets of cæca. The mid-gut, therefore, has now developed into a
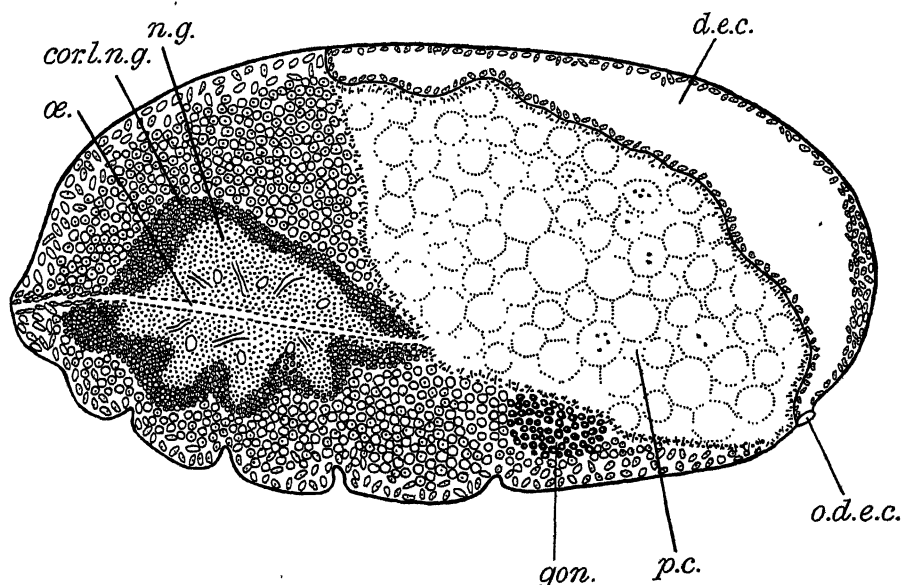
FIG. 19—Reconstructed sagittal diagram to the same scale, showing the completion of the dorsal excretory canal. *cor.l.n.g.*, cortical layer of the nerve ganglion; *d.e.c.*, dorsal excretory canal ; *gon.*, rudiment of gonad ; *n.g.*, nerve ganglion; *o.d.e.c.*, permanent opening of the dorsal excretory canal ; *œ*, œsophagus stomodæum ; *p.c.*, posterior diverticulum of the yolk mass.

median sac with paired cæcæ anteriorly and posteriorly, and a pair of diverticula in the middle region. A protruberance of the median sac, however, grows forwards a small distance above the nerve ganglion, disappearing in later states on account of the growth of musculature in the rostrum.

FURTHER DEVELOPMENT OF GENITAL ORGANS

Stage P (67¾ hours)—In this stage the group of cells that become detached from the lower edge of the mesoderm become more defined and compact so that the genital organs appear like a pair of ovoid shaped

bodies on the ventro-lateral surface of the mesoderm, fig. 18 (a). It is worthy of note that in the centre of the developing gonads a few cells are found to stain more deeply than the neighbouring cells, and the nuclei of these cells are considerably larger than those of others. These cells may represent the ova of the adult, being similar in appearance to the ova found in the gonad of the nymph.
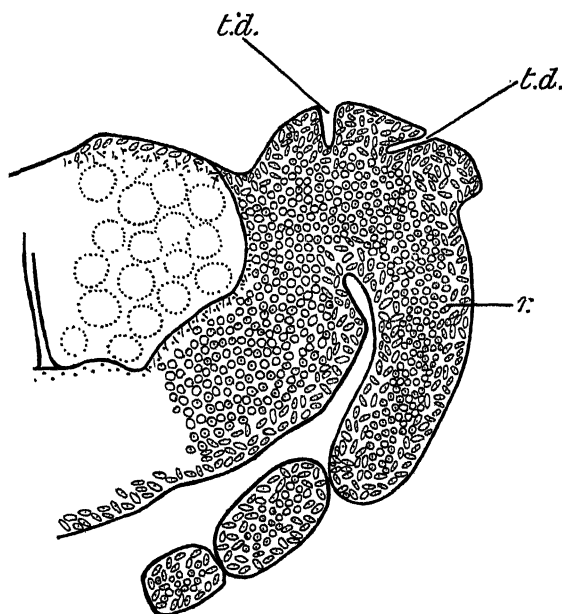

FIG. 20—Lateral sagittal section showing the development of tracheal depressions on the bent rostrum of the embryo. *r.*, rostrum ; *t.d.*, rudiments of trachæa

DEVELOPMENT OF THE RESPIRATORY SYSTEM

Stage Q (72 hours)—The rostrum shows a tendency to bend downwards at right angles to the body of the embryo, so that the cheliceræ come into contact with the first pair of larval legs. On the dorsal side of this rostrum, a little laterally in relation to the region where the pedipalpi join the main body of the embryo, two pairs of tracheal invaginations arise. Fig. 20, which is a sagittal section towards the left side of the embryo, shows the two tracheal invaginations on that side, the anterior one having a rather oblique course, while the posterior one follows a vertical course. The region, where the tracheal depressions arise, corresponds to the position of the lateral stigmata of the adult animal.

Stage R (90 hours)—These depressions deepen further, and in the course of development assume the form of definite canaliculi. The

anterior pair, however, seems to disappear. Fig. 21, which is a sagittal section towards the left side, shows the posterior canal on that side bifurcating and having the form of the letter Y, lying in a sagittal plane, with the single leg pointing forwards to open as the stigmata.

DEVELOPMENT OF THE " SALIVARY GLAND "

At the same time, in the neighbourhood of the opening of the tracheæ, a few ectodermal cells become differentiated, fig. 21. These cells which are
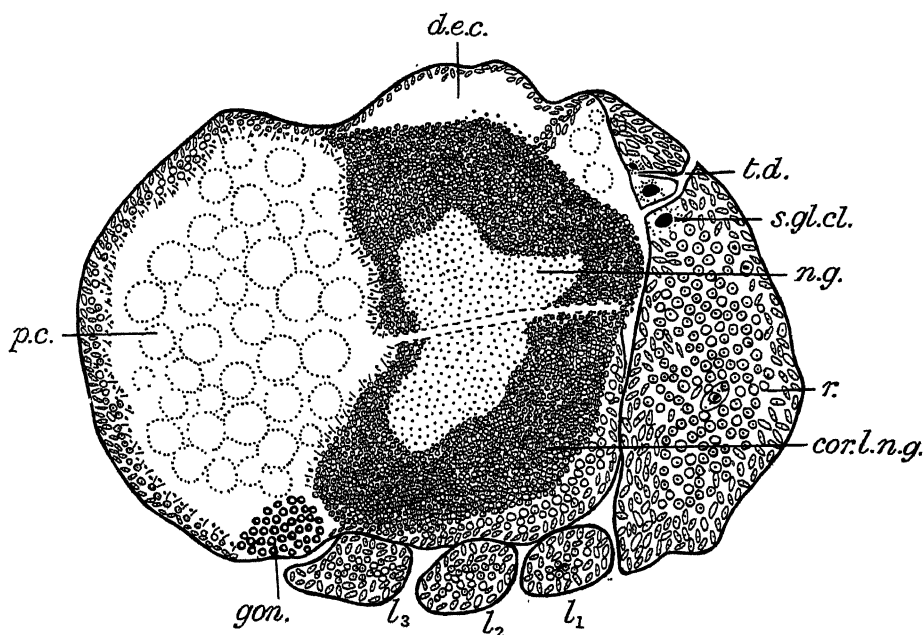

FIG. 21—Lateral sagittal section of an embryo a few hours before hatching, showing the further development of the tracheal system and the origin of the " salivary gland " cells. Abbreviations as in the three previous figures. In addition : *s.gl.cl.*, salivary gland cell

considerably larger in size than the rest of the ectodermal cells, contain large and ill-defined nuclei. At this stage of development, a few cells have begun to sink downwards and are found in the neighbourhood of the tracheal canals, as shown in fig. 21. They, however, appear to correspond to the eosinophilous salivary gland cells of the adult. A few more such cells occupy the edge of the rostrum, but show a tendency to sink down into its musculature in the newly hatched larva.

Ducts of any description have not been observed and, therefore, it is doubtful whether the " salivary gland " is in any way connected with the

function of the digestive system of the animal. *Cheyletus eruditus* when attacking its prey (*Tyroglyphus longior*) is observed to pierce the cuticle of its victim with the stylet-like cheliceræ and by powerful pumping motions of the pharynx effects the transference of the body juices of the victim to its own gut. It is noticeable that the prey becomes completely paralysed a few moments after capture, probably because some poison secreted by the large " salivary gland " is injected. In view of the absence of a duct leading from the gland to the gut and of the above facts, it is possible that this is more in the nature of a poison gland than a salivary gland. Further, these cells correspond to the poisonous dermal glands of other arthropods, particularly the Crustacea. The food of *Cheyletus* is the juices from *Tyroglyphus longior* and therefore of easily digestible nature. Even in the absence of an anus, *Cheyletus* shows a remarkable departure from other animals. It carries out its excretory function by the dorsal excretory canal, there being in consequence of feeding habits practically no solid material to be excreted by an anus. The periodical discharge of the dorsal excretory canal is found to consist of droplets of some liquid, which, when dry, form crystalline bodies, probably urates. The transference of the waste material from the mid-gut is probably through the tunica propria, which lies in close contact with the food in the mid-gut, and which must therefore be permeable to the urate material in liquid form. On account of the peculiarity of the alimentary system and the " chemical food " of *Cheyletus*, one is led to assign to the " salivary gland " a different function from that of digestion.

FINAL STAGE : EMERGENCE OF THE HEXAPOD LARVA

Stage S (96 hours)—At about 96 hours after oviposition, the embryo hatches out as a hexapod larva from the egg shell, which is the second one (deutovum), the first shell having been cast off at about 63 hours. The shell, from which the embryo hatches out, splits open into two halves, the ventral side of which still remains intact. The larva wriggles out of the shell and immediately starts moving on its six legs.

After the emergence of the larva, the rostrum straightens out, but the pedipalpi and the cheliceræ still remain rather closely packed together. The cuticle of the larva is found to be very delicate.

In serial sections, the larva is seen to contain in its cæca the yolk globules on which it still apparently subsists. They, however, disappear when the larva begins preying on *Tyroglyphus*, the gut being then filled with the same fat material with which the adult animal is filled in life.

The " salivary " cells have now come to occupy the middle of the rostrum near the region of the attachment of the pedipalpi and the chelicerae to the body of the larva. The arrangement of these cells is rather symmetrical, there being two rows of three each surrounded by the muscular constituents of the rostrum, fig. 22.
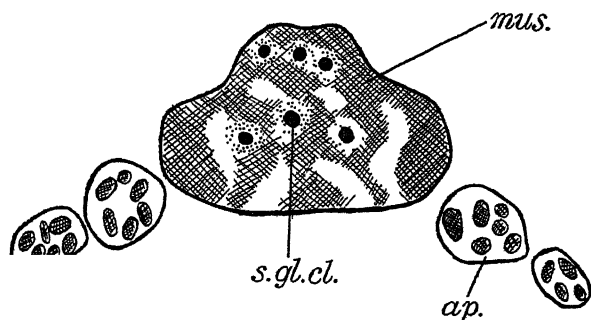

FIG. 22—Transverse section through the region of the rostrum of a young larva 3 hours after hatching. The " salivary gland " cells have here sunk into the middle of the rostrum in the musculature

The gonads have now attained a mid-ventral position and have become fused to form a single ovary near the last pair of legs.

Subsequent stages of the larva do not show much anatomical change as the larva very much resembles the adult morphologically, except for the absence of a fourth pair of legs. These make their appearance after a series of ecdyses preceded by periods of rest.

GENERAL REMARKS

The germ layer formation in *Cheyletus eruditus* shows a typically arachnidan proliferation of endoderm cells. The formation of the mesoderm bands by overgrowth of the mid-ventral region by the lateral region of the blastoderm is similar to that which occurs in some of the insects, *e.g.*, Honeybee (Nelson, 1915). The Acarina as represented by *Cheyletus*, are in this respect probably intermediate between Arachnida and Insecta.

The huge single nerve ganglion though apparently primitive in structure, has a highly specialized development. The number of lobes of the ganglion definitely corresponds to the number of early segments of the embryo, which are represented by the appearance of coelomic cavities in the mesoderm which disappear totally in a later stage.

Even the abdomen shows indistinct traces of metameric segmentation which later completely disappear leaving no indication of it in the adult.

Thus it is seen that though Acarina, as a group, shows no adult segmentation of the arthropodan type, yet in the early embryology segmentation is apparent, though later it disappears. It is probable that the parasitic nature of this animal has definitely given rise to characters most suitable for its mode of life.

The alimentary tract with its related organs, viz., the " salivary gland " and the dorsal excretory organ, shows the adaptation of characters according to the mode of life the animal leads. The development of a capacious mid-gut with its cæca corresponds to the mode of feeding and type of nutrition. As already pointed out, *Cheyletus* sucks in the juices of the victim by the pumping action of the pharynx, the juices being sent directly into the mid-gut for absorption. It is probable that by the muscular activity of the body of the animal, the cæca contract and relax for the drawing in of the liquid food, suction being facilitated by the absence of an anus. In the act of feeding the mouth is plunged into the body of the victim and thus the alimentary system as a whole becomes practically air-tight. Transference of waste material from the mid-gut to the obsolete hind-gut, which here takes the form of a dorsal excretory canal, is probably carried out by osmosis, the tunica propria being permeable to the transference of soluble urates.

It has already been shown that the " salivary gland " has no direct visible communication with the gut, and that it probably has a poison-producing function in life. Further development of the gland shows great resemblance to the mode of development of the poison glands of the Araneæ, with which the Acarids as a whole have a close relationship. According to Schimkewitsch (1886), the poison glands in Araneæ arise as ectodermal thickenings at the tips of the cheliceræ and grow inwards from that point, a condition which is more or less repeated in *Cheyletus eruditis.*

The presence of stigmata in the cephalothoracic region in *Cheyletus eruditus* and the development of the respiratory system must be regarded as a secondary acquisition. Such a condition is found in *Scolopendrella,* where the stigmata are known to occur in the head in an unusual manner.

The blood-vascular system, as such, in *Cheyletus* has not been described in the adult anatomy, as the adult neither possesses a tubular heart of the arachnidan type nor any definite blood-cells floating in the body cavity. But the development shows the presence of certain amœboid cells which float in the gut cavity at an early stage. The origin of these cells has been clearly demonstrated to be mesodermal, but their fate cannot definitely be settled. Still, the presence of these " blood-cells " in the embryonic

stage may show the relationship of *Cheyletus* to other Acarids, *e.g.*, *Gamasus fucorum*, where the larva possesses a heart in the form of a rounded organ at the posterior end of the abdomen. Even for *Gamasus*, however, the compact form of the heart shows the reduction undergone by the whole body in Acarina. In *Cheyletus* this appears to have become very degenerate, and therefore no organized circulatory system is present.

The hexapod larva of *Cheyletus eruditus* and other Acarids shows a ' strong general resemblance in organization to the adult, with the exception of the larva of *Phyloptidæ* in which there are only two pairs of larval legs. In the larva of *Oribatidæ*, the respiratory system is entirely wanting.

As regards the larval integuments, it must be pointed out that although there are two larval envelopes, of which the second one is called by Claparède the " deutovum," what was described by Kramer as the horny apparatus for splitting the envelope is not sufficiently evident. The figures which Kramer drew do not clearly depict the tooth-like processes he described in his paper. The second larval shell is simply cast off by splitting into two halves, probably by the straightening of the bent rostrum.

The time-table of development will show two long periods of about 18 hours each, during which the development seems to be slackened, especially in the case of the earlier one. At this period, according to Kramer, the embryo appears to conserve all its energy for the breaking up of the first egg shell. The emergence of the larva from the second egg shell is preceded by a second period of apparent rest, but the development does not come to a standstill as in the previous one. Post larval ecdyses take place after inert periods of 3, 3, and 4 days respectively before succeeding ecdyses. Thus the organism seems to conserve its energy by periods of rest for casting off the integuments.

SUMMARY OF NEW POINTS IN THE EMBRYOLOGY OF THE GROUP ACARINA

The single nucleus of the ovum rises to the surface of the yolk and by dividing gives rise to a single layer of cells forming the blastoderm. This becomes differentiated into two thickenings forming the lateral plates, and a median one forming the middle plate.

Endoderm cells are proliferated into the yolk from the ventral region of the blastoderm—that is, the ventral plate—while the middle plate gives rise to the mesoderm. The lateral plates together with the thin strip of blastoderm on the dorsal side give rise to the ectoderm. The middle plate is overgrown by the lateral plates which meet in the mid-ventral line beneath it.

The stomodæum arises as an ectodermal invagination in the anterior region of the germ band. Nerve cells arise from the ectoderm of the ventral portion of the germ band in the head-lobe.

The stomodæum pierces its way through the nerve ganglion.

The development of the nerve ganglion shows five pairs of rudiments in the form of lobes.

Early segmentation of the body is seen in the cephalothoracic region and less distinctly in the abdominal region.

Five pairs of thickenings of the germ band arise, which later develop into the larval appendages.

The dorsal excretory canal has been demonstrated to be purely ectodermal in origin, and is suggested to be the homologue of a proctodæum which fails to establish a connection with the mid-gut.

The yolk sac gives rise to the mid-gut, which acquires a covering of endoderm through the migration of yolk cells to the surface.

Longitudinal and transverse septa develop and divide the yolk sac into paired cæca.

The absence of an anus both in the embryonic stages and in the adult has been proved beyond doubt.

The gonads arise from the lower edge of the mesoderm blocks as a pair of thickenings which later fuse into a single median ovary.

Amœboid " blood cells " arise from the splanchnic layer of mesoderm in the abdominal region.

The respiratory system originates on the lateral sides of the rostrum as two pairs of ectodermal depressions, of which the anterior pair later disappears.

" Salivary gland " cells arise in association with the tracheæ. They are proved to be more akin to the cells of poison glands.

A hexapod larva, resembling the adult, emerges out of the second egg shell.

Two pre-larval periods of rest of 18 hours each are observed before the casting of the first and second egg shells in correspondence with the post-larval inert periods of 3, 3, and 4 days respectively before succeeding ecdyses.

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Hypophysectomy of Birds

IV—Plumage changes in Hypophysectomized Fowls

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[PLATE 17]

1—INTRODUCTION

We have previously described the effects of hypophysectomy on the reproductive organs and comb of the fowl. It remains to consider the plumage changes. Castration of the normal male fowl causes no plumage changes except slight lengthening of the feathers. Ovariectomy of the hen, however, results in reversal of the plumage to the male type. The plumage of the normal male is thus of the asexual or neutral type found in the gonadectomized bird of either sex (Goodale, 1916; Pézard, 1918). We expected, therefore, to find little change in the plumage of the hypophysectomized cock, in spite of the virtual destruction of the testis (Hill and Parkes, 1934), but a definite reversal of the hen's plumage to the male type. The changes observed were actually very different; Brown Leghorn cocks have shown remarkable plumage changes, the hens much less obvious ones. We are convinced that the chief factor concerned in these plumage changes is thyroid deficiency. It is well known that there is an intimate relation between hyper- and hypo-thyroidism and plumage type (see Greenwood and Blyth, 1929). Thyroid-ectomy causes inability to produce the black pigmentation on certain areas of the body, especially in such breeds as the Brown Leghorn, and increases the fringing of the feathers due to lack of barbules. Changes such as these are the most obvious feature in the hypophysectomized fowl, and since we have demonstrated that they can be corrected by the administration of thyroxine, there seems little doubt that the thyroid atrophy which is known to follow hypophysectomy in mammals also occurs in birds and that this is the decisive factor in the post-hypophy-sectomy plumage changes.

The study of plumage has now become a specialized branch of develop-mental physiology, largely owing to the researches of the Chicago workers

in America, of Zawadowsky in Moscow, and of Greenwood in this country (see Lillie, 1932; Lillie and Juhn, 1932; Zawadovsky, 1927; and Greenwood and Blyth, 1929, for comprehensive papers with references). In the circumstances we have hesitated to attempt any complete analysis of the effects of hypophysectomy on plumage; such an analysis will clearly demand detailed knowledge of the subject. Our aim in giving the present description is to indicate the general nature of the changes, in the hope that they will be fully dealt with by other workers.

2—MATERIAL AND TECHNIQUE

The birds were hypophysectomized as previously described (Hill and Parkes, 1934). Two of the five fowls recorded in this paper are still alive (August, 1934) and in good condition (HF40 and HF68), but the characteristic changes in the comb and wattles make it reasonably certain that the anterior pituitary is completely removed. Histological examination of the sellæ turcicæ of the other three showed definitely that removal of the anterior lobe had been complete, though most of the posterior lobe was present in two, HF52 and HF60. Under these conditions, as we have already shown, endocrine activity of the gonads ceases very soon. All of the birds described here were injected with either cortin or anterior lobe extract for the first few days after operation, but this cannot have affected the subsequent plumage type. Three of the birds were injected at a much later date with gonadotropic extracts or thyroxine, but the changes discussed here are purely those occurring in the untreated hypophysectomized bird.

The fact that no changes occur in the fully-formed feather makes it necessary in the ordinary way to pluck an area or to wait for natural moulting, so that the effect of the new conditions can be observed on a developing feather. In the hypophysectomized bird, this problem is much simplified by the severe moult which follows about a fortnight after operation. The male Brown Leghorns shed practically every feather within 2–4 weeks of operation and the field was clear for the new crop to develop under the new conditions. Further, the hypophysectomized fowl, like the capon, seems to be perpetually moulting and regenerating odd feathers. At the time of operation, or immediately afterwards, samples of the existing plumage were obtained to act as control material on whatever might regenerate afterwards. Specimen feathers were taken from the breast, legs, upper and lower neck, cape, wing coverts and saddle.

R 2

Of the five fowls described here three were cocks, two Brown Leghorns and one Rhode Island Red, and two hens, of Brown Leghorn type, but possibly from an impure strain. They were from 73–127 days after hypophysectomy at the time of making the final observations on the plumage, and all had grown a complete new set of feathers after the post-hypophysectomy moult. In HF40 a second crop of post-hypophysectomy feathers were available for examination, following the plucking of an area of the first crop.
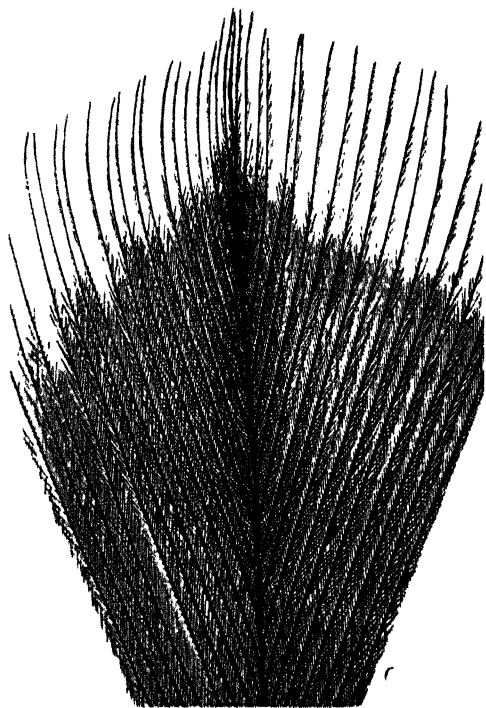

Fig. 1—Normal breast feather of Rhode Island Red cock, showing lack of fringing

3—Changes in the Cock

HF36. *Rhode Island Red Cock*—No colour change was observed in this bird. The whole plumage, however, was much more glossy than previously, owing, as could be seen from examination of individual feathers, to a great increase in the fringing of the feathers caused by lack of barbules. The feathers which had previously shown a solid centre core, *i.e.*, neck and saddle hackles, wing coverts and cape feathers, now had the solid part so much reduced that the feather was nearly all fringe.

The feathers were also narrower and more pointed. Feathers which had not previously been fringed, *i.e.*, those on the breast, were now fringed in many cases, figs. 1 and 2. This increase of fringing is a typical thyroid deficiency effect.

HF40. *Brown Leghorn Cock*—This bird was a well-marked specimen of the breed at the time of operation, and showed no brown flecking on the black ventral feathers. When the new breast feathers began to come
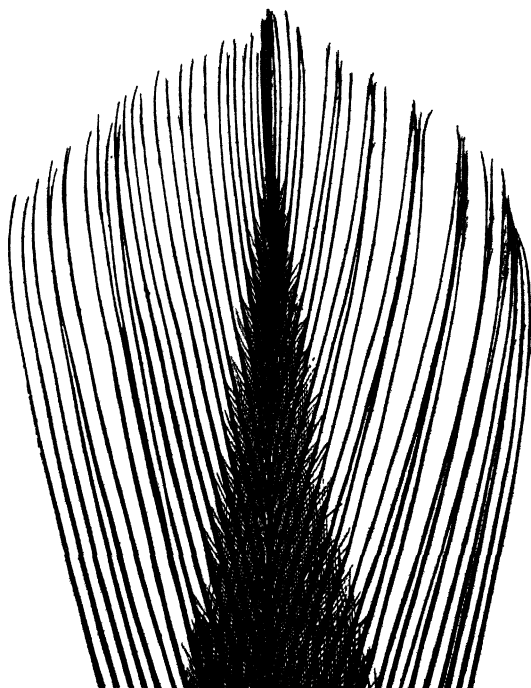

FIG. 2—Breast feather of hypophysectomized Rhode Island Red cock, showing loss of barbules resulting in fringe

through the skin, in the area which had been plucked for the tracheotomy, it was immediately seen that the new plumage would be different. High up on the breast, on the under side of the neck, the new feathers were chocolate brown with or without a black tip, while lower down black predominated, but was freely splashed with brown. These ventral surface feathers were not fringed at this stage. Later on, newer feathers were less black and in many cases showed fringing which turned the rich brown of the solid feather into glossy gold, as in the saddle hackles. An area of the breast replucked two months after operation produced a

second crop which was nearly all brown and gold, a condition also found in odd new feathers which grew later than the main post-hypophysectomy crop, fig. 6, Plate 17.

At 125 days after operation the plumage was entirely different from that of the normal Brown Leghorn cockerel. There were no wholly black feathers, except a few odd ones on the lower breast and in the tail. The neck hackles were practically devoid of black and the general effect here was of unbroken golden-brown at the back of the head, shading down to orange at the base of the neck. The individual feathers, in addition to the loss of the black core usually found in the lower ones, showed a great reduction in the amount of solid feather and a corresponding increase in the amount of fringing, fig. 8, Plate 17.

The feathers of the ventral surface varied from small solid brown ones high up on the under neck, through brown with black tips, to black mottled with brown on the lower breast and legs. Amongst these, the later feathers with the extensive glossy gold fringe stood out markedly from the rest of the ventral plumage. From what we have observed of the feather succession, it seems likely that in time the ventral surface will become entirely devoid of black, and that few of the feathers will have much solid part. The breast feathers were longer and more pointed than is normal. The higher wing coverts, covering the anterior point of the wing, which are small solid black feathers in the normal bird, had also lost their black pigment, except in the basal fluff, and the solid part of the feather was reduced to a small red core, the remainder being glossy brown fringe, fig. 7, Plate 17. The lower wing coverts and the cape feathers had changed little, except that they had less black and the central core of solid feather was reduced by an extension of the fringing down the side. The wing primaries appeared unchanged. The saddle hackles had lost the small amount of black previously present at the base of the solid core, and the whole feather was narrower, owing to reduction of the solid core to a small patch above the fluff. The fluff feathers covering the belly and sides, previously black, were indiscriminately banded brown and black.

HF68. *Brown Leghorn Cock*—The plumage changes observed in this bird were essentially similar to those in HF40, except that, right from the first regeneration of feathers in the area plucked for tracheotomy, the ventral surface feathers came more wholly brown and more fringed than in HF40. At 80 days after operation, the ventral surface feathers were predominately brown, and more of the feathers had the glossy gold fringe than in HF40. All over the body the changes gave the impression

of occurring more rapidly than in HF40. The bird is distinguished by the presence of one albino feather among the neck hackles.

Sebright Bantam Cock—As recorded previously, an adult Silver Sebright cock was successfully hypophysectomized and lived in good condition for 39 days, when it died as the result of an accident. This bird moulted very heavily and became almost bare in the third week after operation. At the time of death the new crop of feathers was sufficiently grown to show that an extensive change had occurred in the plumage. The neck hackles, for instance, were coming through pure white, and there is no doubt that, if this bird had lived, the characteristic change to male type plumage which is found in this breed after castration (Morgan, 1919) would have occurred. How far this change-over would have been complicated by thyroid deficiency is difficult to say.

4—CHANGES IN THE HEN

HF52. *Brown Leghorn* (? *Impure*) *Hen*—Feathers regenerated after the post-operation moult and before experimental treatment was begun were as follows: the ventral surface feathers showed no change in shape, and no change in colour except a slight darkening. There was no fringing of the breast feathers such as occurred in the males. The neck hackles, however, underwent a definite change to what was superficially the male type. They were longer and had a marked fringe. Some of the earlier growing lower ones had a black core at the top, but this was discontinued as growth proceeded, fig. 9, Plate 17. The higher ones were glossy gold all through and consisted mostly of fringe. Except for being slightly shorter, they were identical with similarly placed ones in HF40 and HF68. Replacement treatment was started on this bird before the new neck hackles were fully grown, but it seems likely that this bird would have acquired a full set of hackles similar to those found in HF40. The post-operative dorsal plumage was very luxuriant, and from the cape to the tail coverts the feathers were fringed without exception and were bright brown in colour in place of the previous drab stippled greyish brown. The feathers as a whole were longer and more pointed than before operation. This was well shown in the case of the saddle hackles, which, in place of being short and blunt, were now longer and wedge-shaped. They were noteworthy in being whole-coloured and quite devoid of the characteristic female stippling, but even so they showed only an approximation to the male type, fig. 10, Plate 17. This female differed from the males in that the more recent feathers showed no sign of greater alteration than those growing immediately after operation.

HF60. *Brown Leghorn Hen*—This was a lightish-coloured bird, and the change-over to the rich brownish colour in the dorsal feathers was even more marked than in HF52. Otherwise the reaction after hypophysectomy was very similar to that in HF52, *i.e.*, no change in the breast feathers, reversion of the neck hackles in the direction of the hypophysectomized male type, and elongation and fringing of the other dorsal feathers without any close approximation to the male type.

5—DISCUSSION

The plumage changes described above as following hypophysectomy of the cock seem to be so similar to those which follow thyroidectomy that they may safely be attributed to thyroid deficiency. Externally, therefore, a hypophysectomized cock is similar to a thyroidectomized cock, or allowing for the comb change, to a thyroidectomized capon. No changes have so far been observed which could be definitely ascribed to a direct effect of pituitary deficiency on the feathers, but since the plumage type of these birds is as yet far from stable, it is possible that such changes will appear in the course of time.

The results on the hens are not so easily explained. From the atrophy of the ovary which follows hypophysectomy, it could have been expected that the change to the male (asexual) type would have occurred. The coincident thyroid deficiency would presumably have prevented the development of certain features, such as the black breast feathers in the Brown Leghorns, but the typical lengthening and fringing of the male feathers would have been expected. Except for the neck hackles, which became very similar to those of the hypophysectomized male, the feathers of the two hypophysectomized hens did not show any close approximation to what would have been expected in a thyroidectomized, ovariectomized hen. Sections of the sellæ turcicæ of these two birds showed no trace of anterior lobe tissue, and no obvious explanation of the behaviour of the hens presents itself.

One point of wider implication may be made from these experiments on birds. Greenwood, from his experience of partial thyroidectomies, concludes that quite a small amount of thyroid activity is sufficient to maintain the black breast pattern of the Brown Leghorn cock. In view of this it may be concluded that a total thyroid deficiency, so far as plumage maintenance is concerned, is caused by hypophysectomy in the bird. In this respect, therefore, it would appear that the thyroid is similar to the gonad, in being entirely dependent on the pituitary.

·We are greatly indebted to Dr. A. W. Greenwood and Dr. G. H. Faulkner, who examined these birds and gave us much information about plumage.

6—SUMMARY

The plumage changes after hypophysectomy have been studied by observation of the feathers regenerated in plucked or moulted areas.

Hypophysectomy of the Brown Leghorn cock results in the loss of most or all of the black pigment from the feathers of the under-neck, breast and legs. The later-growing feathers, particularly, are usually devoid of black and may be extensively fringed. The new plumage over the rest of the body is characterized by loss of black pigment and increased fringing due to lack of barbules.

These changes are so similar to those which follow thyroidectomy that they may reasonably be supposed to be due to thyroid deficiency, which is well known to follow hypophysectomy in mammals.

In the hens, fringing and elongation was observed in the dorsal feathers as well as loss of the typical female stippling, but apart from the neck hackles, which became very similar to those of the cocks, no close approximation to the male type was observed.

Hypophysectomy of Birds

V—Effect of replacement Therapy on the Gonads, Accessory Organs and Secondary Sexual Characters of Hypophysectomized Fowls

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1—INTRODUCTION

In the course of making the necessary replacement therapy to carry the birds over the early post-hypophysectomy period, certain effects of anterior lobe extracts on the newly hypophysectomized fowl were observed. These observations were re-enforced by a long duration experiment with anterior lobe extracts, and by other experiments designed to show the effect of sex hormones and also of thyroxine on hypophysectomized fowls. The account given below concludes the description of our work on hypophysectomized fowls.

2—MATERIALS AND TECHNIQUE

All techniques were as previously described.

Hormone Preparations—The anterior pituitary preparations were neutralized alkali preparations of acetone-desiccated ox or horse anterior lobe, that of the former being a dry powder obtained by alcohol precipitation, which was put into solution for injection as desired. The urine of pregnancy preparation was made by the benzoic acid adsorption method and had a rabbit ovulation unit (Rb.U.) of about 0·5 mg. The male hormone preparation was an oil solution containing about 8 capon units per cc. Oestrin was given in the form of œstrone benzoate. The thyroxine was a synthetic crystalline preparation.

Plumage Changes—Where it was desired to investigate the effect of treatment on the post-hypophysectomy plumage the usual technique of plucking a small area some days beforehand was adopted.

3—Effect of Anterior Pituitary Preparations Given
Immediately after Hypophysectomy

(*a*) *Testis Histology*—The testes of HF54, which received about 200 Rb.U. of gonadotropic substance in the five days after operation, were obtained 15 days after hypophysectomy. They were appreciably less atrophic than those of HF42, which received no anterior lobe extract, obtained 14 days after operation. A few spermatozoa were still present as compared with the absence of stages later than spermatocytes in HF42. The testes of HF70, receiving 500 Rb.U. of gonadotropic substance in the 5 days after hypophysectomy were obtained at 20 days after operation. The testes were less atrophic histologically and rather heavier than would have been expected at this stage without injection (Hill and Parkes, 1934, *b*). It was not found possible, however, to maintain spermatogenesis by the continuous administration of gonadotropic hormones. HF71 was injected daily for 34 days with 100 Rb.U. of anterior lobe extract, starting on the day of operation. At the end of this time a fragment of the left testis was removed and the injections were stopped. Histological examination showed that the testis was in practically the same condition as that of HF37 at 35 days after operation (Hill and Parkes, 1934, *b*), the only difference being that the tubules were rather larger and contained more debris. The bird died at 50 days after operation, when the testes weighed 0·58 gm. It must be concluded, therefore, that injection of anterior pituitary extract of this kind may slightly delay the initial stages of atrophy but completely fails to maintain spermatogenesis after hypophysectomy.

(*b*) *Comb*—It was shown (Hill and Parkes, 1934, *b*) that the comb of birds not given anterior lobe extract after hypophysectomy began to shrink immediately. In birds which received anterior lobe extract for a short period immediately after hypophysectomy, the comb was observed to undergo a temporary increase in size before the shrinkage began. This applied to both males and females and to all the 10 birds observed (Table I). This increase amounted to as much as 40% (L × H) in some of the birds. In view of these observations we confidently expected to be able to maintain the comb after hypophysectomy by injection of anterior lobe extract. This expectation was not realized. In HF71, injected daily for 34 days, the comb began to atrophy after the initial rise, in exactly the same way as in the birds receiving only five daily injections, fig. 3. This result shows that not only is spermatogenesis not maintained by this type of extract, but that the internal secretory activity of the testis also disappears in spite of injection. It should, however, be

recorded that HF71 crowed lustily for more than a month after operation, twice as long as the record for birds receiving only temporary injection. There was no inhibition of the post-hypophysectomy moult.

TABLE I—CHANGES IN COMB SIZE IN BIRDS INJECTED WITH ANTERIOR LOBE EXTRACT IMMEDIATELY AFTER HYPOPHYSECTOMY

Number of bird	Days injected after hypophysectomy	Size of comb (cm)	
		At hypophysectomy	Maximum during injection
HF52	6	7·1 × 3·1	7·2 × 3·7
HF60	4	5·9 × 3·3	6·3 × 3·6
HF63	6	6·0 × 3·3	6·8 × 3·7
HF64	6	6·5 × 2·9	7·4 × 3·6
HF65	6	6·7 × 4·2	7·3 × 5·3
HF66	6	6·9 × 3·9	7·0 × 4·2
HF54	5	11·5 × 7·9	11·5 × 8·0
HF68	5	13·2 × 7·2	13·4 × 7·9
HF70	5	12·9 × 6·8	13·2 × 6·9
HF71	5	14·0 × 8·5	14·6 × 8·5*

* Injections were continued in this bird but the maximum measurement during the first 5 days period is given here for comparison with the others.
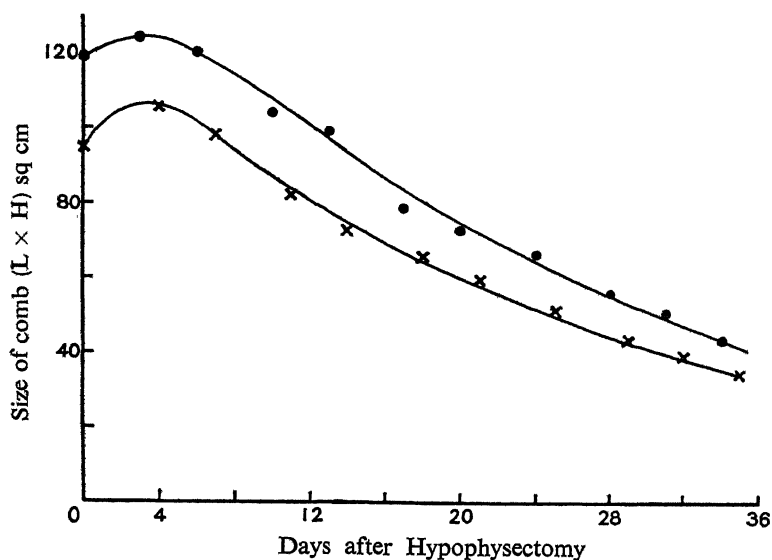

FIG. 3—Lack of effect of continuous injection of anterior lobe extract on the atrophy of the comb of the hypophysectomized cock ...... HF71, given anterior lobe extract daily for 34 days after hypophysectomy. × × × HF68, given anterior lobe extract for 5 days only after hypophysectomy.

(c) *Plumage*—The anterior lobe extract given to HF71 was strongly thyrotropic, 4 mg for 5 days being sufficient to double the weight of the thyroids of the immature guinea-pig. HF71 received 3·4 gm of this extract in 34 days, and it might reasonably have been expected that the thyroids would have been maintained sufficiently to prevent the plumage change described in Part IV. In actual fact, however, the breast feathers showed loss of black pigment and appearance of fring-ing, in exactly the same way as in the uninjected cocks. There was no sign that injections delayed even for a short time the change over of the plumage, fig. 4. The explanation of this failure to maintain the breast plum-age is not clear. The experiments described below make it reasonably certain that the loss of black pig-ment, etc., is due to thyroid deficiency, and it must be supposed that the thyroid atrophy was not even retarded, which is curious in view of the very large amount of thyrotropic substance administered.

4—EFFECT OF GONADOTROPIC EXTRACTS IN HYPO-
PHYSECTOMIZED FOWL OF LONG-STANDING

(a) *Testis Histology*—We attempted to restore sper-matogenesis in the testis of a long-standing hypophy-sectomized cock. Four months after hypophysectomy and two months after the last administration of gonado-tropic hormone, a piece of the left testis of HF36 (see Hill and Parkes, 1934, *b*) was removed by operation. During the next 8·days, rather more than 5000 Rb.U. of gonadotropic substance from horse pituitary was given. The bird was then killed. Histological examina-tion of the piece of testis removed before injection showed a condition very similar to that of HF37, 35 days after hypophysectomy, except that the tubules were rather smaller and completely occluded by syncytium, while the inter-tubular tissue was rather less in amount.

FIG. 4—Breast feather of HF71, showing loss of black pigment in spite of daily in-jection of anterior lobe extract.

Injection appeared to increase the diameter of the tubules slightly and to produce a small lumen in many of them, but no appreciable advance in spermatogenesis was observed. Some slight endocrine activity had apparently been caused, because the epithelium of the vas deferens was much more healthy-looking than that of HF36, being columnar with large nuclei and a definite basement membrane, compared with the dense

cuboidal lining of the vas of the untreated hypophysectomized bird. The actual size of the testes cannot have been increased much, since the weight at autopsy was only 0·53 gm.

(b) *Ovary*—HF52, 113 days after hypophysectomy, which had not received further gonadotropic hormone since the sixth day after operation, was given 5000 Rb.U. from horse pituitary over 5 days.   At the beginning of injection a laparotomy was made and the ovary was seen to be small with no follicles above about 2 mm diameter. After the fifth injection the bird was killed. The ovary was slightly enlarged, but no radical change was observed. The oviduct was much increased in size, as compared with untreated hypophysectomized birds, measuring 45 cm in length, but this may partly have been due to previous œstrin treatment.

(c) *Comb*—Five weeks after hypophysectomy the comb of HF36 had shrunk to 6·5 × 2·9 cm, and, by analogy with other birds, it was assumed that testis atrophy was far advanced.   The bird was then given 400 Rb.U. of gonadotropic substance from ox pituitary daily for 8 days. There was no change whatever in the size of the comb.   10,000 Rb.U. from horse pituitary was then given over 17 days. The behaviour of the comb during this time was rather remarkable. During the first 10 days it increased by about 60% (L × H).   At the same time the comb became bright red, the papillæ developed, and generally it lost the appearance of the capon type. This development, however, did not continue, and, moreover, the existing development was not maintained. At 17 days the comb had shrunk practically to its previous size, and injections of anterior lobe extract were replaced by 20 Rb.U. daily of urine of pregnancy. At the end of this time the comb was still shrinking slightly and injection was discontinued. The total result of this experiment therefore, was that the atrophic comb had been caused to enlarge appreciably by extract of horse anterior lobe, but that the development could not be maintained. This result appears to afford another example of the loss of sensitivity to anterior lobe preparations to which Collip (1932) and McPhail (1933) have called attention.

Two months later, an approximately similar daily dose, which as recorded above failed to stimulate spermatogenesis, failed also to produce any change in the comb.

HF65 was given 100 Rb.U. from ox pituitary starting 23 days after hypophysectomy, when the comb was still decreasing in size. By the fourth day, the size of the comb had increased by about 20%; this increase,

again, was not maintained by further injection, and the comb continued to atrophy.

HF52 received 5000 Rb.U. of gonadotropic substance from horse pituitary, for 5 days starting 113 days after hypophysectomy, but failed to show any appreciable development of the comb.

5—EFFECT OF THYROXINE ON THE PLUMAGE OF THE HYPOPHYSECTOMIZED FOWL

Since the plumage changes in the Brown Leghorn male, following hypophysectomy, are entirely similar to those following thyroidectomy, and since the administration of thyrotropic extracts failed to avert this change, it seemed highly desirable to ascertain the effect of thyroxine treatment. This was carried out on HF68, which, apart from the 5 days immediate post-operation treatment with anterior lobe extract, had received no further treatment up to 66 days after operation. The plumage at this time was as described on p. 206, the ventral feathers being predominately brown, with well-marked fringing. Two weeks previously a small area on the breast had been specially plucked and new brown feathers were showing. The bird was then given 1 mg crystalline thyroxine in aqueous solution per day for 2 days. Within 48 hours of the first injection the growing feather germ was showing black, and for 9 days the new feathers grew black. Afterwards the germ reverted to brown. When two-thirds grown, all these new feathers presented a uniform appearance. There

FIG. 5—Breast feather of HF68, showing wide black bar resulting from two consecutive daily injections of 1 mg thyroxine.

was a golden-brown fringed tip about 5 mm long, and a solid black unfringed bar about 2 cm wide below, while the lower part of the feather was of the usual chocolate brown colour found in the hypophysectomized male, fig. 5. This experiment shows with reasonable certainty that the change in the breast plumage after hypophysectomy is due to thyroid deficiency.

It seemed possible, as mentioned above, that the failure of the hypophysectomized hen to show significant colour changes towards the male type might be due to the same thyroid deficiency. We were unable to confirm this supposition by thyroxine administration; HF52 received one dose of 0·5 mg and a week later three daily doses of 1·5 mg crystalline

thyroxine, without showing any black in the breast feathers, although the usual black banding of other feathers such as the neck hackles was produced. HF60 was given a very large dose (2 mg per day for 4 days) which killed her. At death a few of the new breast feathers were showing black at the base, but it is doubtful whether this would have been a uniform result.

6—EFFECT OF MALE HORMONE AND ŒSTRIN ON THE COMB OF THE HYPOPHYSECTOMIZED FOWL

HF36 was given 7 capon units of male hormone daily for 5 days, starting at 99 days after hypophysectomy when the comb was stable at $5 \cdot 9 \times 2 \cdot 3$ cm. On the sixth day, an increase in L + H of 6 mm had taken place. 1 unit a day for 5 days was found to give almost exactly the same increase in the comb of a Brown Leghorn capon, and the response of the hypophysectomized bird was therefore poor. Previously, the same bird had been given 1 mg daily of œstrone benzoate without causing any detectable increase in the comb, while a similar amount of the hormone given to an hypophysectomized hen, HF52, also caused no growth in the comb. Since Juhn and Gustavson (1930) obtained only slight indications of comb stimulation in capons and pullets by injection of the œstrus-producing hormone, these results were not unexpected. The failure of HF36 to make a good response to male hormone is more curious, since it is unlikely that the comb of the hypophysectomized bird would be less sensitive than that of the capon. The discrepancy is possibly connected with the fact that HF36 was a Rhode Island Red.

7—SUMMARY

Fowls injected with ox anterior lobe extract for 4–6 days after hypophysectomy all showed a temporary increase in the size of the comb, and, in the male, the atrophy of the testes was slightly retarded. Prolonged injection after operation, however, failed to avert (a) the comb shrinkage, (b) the testis atrophy, or (c) the plumage changes, which follow hypophysectomy.

Attempts to restore the atrophied gonads and combs of hypophysectomized birds by injections of anterior lobe and urine of pregnancy extracts were comparatively unsuccessful.

Administration of thyroxine to a hypophysectomized Brown Leghorn cock restored the breast feathers to normal colour.

The comb of the hypophysectomized cock responded to male hormone, but not to œstrone.

[*Note added in proof*, March 2, 1935—In Part I ('Proc. Roy. Soc.,' B, vol. 115, p. 402 (1934)) we stated that hypophysectomy had not previously been carried out successfully on birds. We were mistaken. In a paper published in 1929,* J. B. Mitchell, working in the Whitman Laboratory of Experimental Zoology, Chicago, described the effect of hypophysectomizing young Brown Leghorns. Using a route similar to that employed by us, he found that " in all cases of complete removal of the anterior lobe, the bird died within 10 days." Sub-total hypophysectomy was not necessarily fatal, and, if only a small fragment remained, led to stunting of the bird, accompanied by delay in assuming the adult plumage and in the attainment of sexual maturity, but specific inhibition of the gonads was not produced. Thyroid changes after partial hypophysectomy were also described, but as these do not seem to have affected the plumage they can hardly have been severe.

This important paper appears to have been singularly overlooked. So far as we can trace it has not been referred to in subsequent papers by the Chicago workers, nor in " Sex Internal Secretions,"† which contains a full discussion of sex hormones and plumage, and of the problem of whether the pituitary is essential for life.]

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DESCRIPTION OF PLATE 17

Fig. 6—(*a*) Breast feather of HF40 ♂ at operation. Original plumage of HF68 ♂ was similar.

(*b*) and (*c*) Breast feathers regenerated on HF40 after operation, showing partial or total loss of black pigment and occurrence of fringing.

(*d*) Feather regenerated in HF68 after operation, similar condition to (*b*).

* Mitchell (1929). ' Phys. Zool.,' vol. 2, p. 411.

† Allen (Editor) (1932). " Sex and Internal Secretions." Williams and Watkins, Baltimore.

FIG. 7—(*a*) Upper wing covert of HF40, at hypophysectomy.
　　　　(*b*) Upper wing covert of HF40, regenerated after hypophysectomy, showing loss of black pigment and appearance of fringing.

FIG. 8—(*a*) Lower neck hackle of HF40, at hypophysectomy.
　　　　(*b*) Lower neck hackle of HF40, regenerated after hypophysectomy, showing loss of black pigment and increase of fringing.

FIG. 9—(*a*) Lower neck hackle of HF52 ♀, at hypophysectomy.
　　　　(*b*) Lower neck hackle of HF52, regenerated after hypophysectomy, showing elongation and increase of fringing.

FIG. 10—(*a*) Saddle hackle of HF52, at hypophysectomy.
　　　　(*b*) Saddle hackle of HF52, regenerated after hypophysectomy, showing change of shape, loss of stippling, and appearance of fringing.

The Kinetics of Photosynthesis

By E. C. C. BALY, F.R.S.

The influence of the various controlling factors on the rate of photo-synthesis is one of the most interesting problems met with in the study of the living plant. If the photosynthetic cycle be considered as a problem in physical chemistry, the three important controlling factors are temperature, intensity of irradiation, and concentration of carbon dioxide in the surrounding medium. During recent years accurate measurements have been made by Warburg and by Emerson of the influence of each of these on the rate of photosynthesis, but no explana-tion of that influence has been put forward.

Of the three controlling factors, the most important is the temperature, since an explanation of the whole process must depend on a complete understanding of the relation between the velocity of photosynthesis and the temperature. It is obvious from the fact that the velocity increases with the temperature that there must be associated with the primary photosynthetic reaction a dark reaction which has a temperature coefficient. This was first recognized by F. F. Blackman (1905), who also proved that when the intensity of illumination is very small the rate of photosynthesis becomes independent of the temperature, a fact which was later confirmed by Warburg (1919). It is evident from

this that the Blackman reaction cannot be the precursor of the primary photosynthetic reaction, and there seems little doubt that it is the restoration to its initial state of the chlorophyll which has undergone some change as the result of the primary reaction (Emerson and Arnold, 1932, *a*).

It follows from this view that when the leaves of plants are irradiated at constant temperature, a photostationary state will be established when the velocity of the Blackman reaction becomes equal to the velocity of the primary photosynthetic reaction. If now the temperature be raised, the velocity of the Blackman reaction will increase and the photostationary state will be shifted towards the side of the photosensitive chlorophyll unit, so that the rate of photosynthesis will be increased. The temperature coefficient of the observed rate of photosynthesis will, therefore, not be the same as that of the Blackman reaction. This is known to be the case and has occasioned some mystification.

In order to define the conditions governing the photostationary state, it is necessary to formulate the two processes, namely, the primary photosynthetic reaction and the Blackman reaction. As the result of experimental work carried out in these laboratories (Baly, 1932) there is little doubt that the primary photosynthetic reaction is a photochemical oxidation-reduction process. If in the first instance this process as applied to chlorophyll be expressed in the simplest terms, the complete primary photosynthetic reaction will be

$$C_{55}H_{72}O_5N_4Mg, CO_2H_2O = C_{55}H_{70}O_6N_4MgH_2O + CHOH,$$
$$\text{Chlorophyll A} \qquad\qquad \text{Chlorophyll B}$$
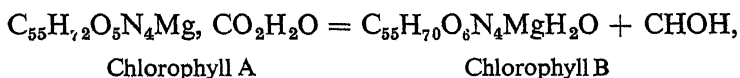
where CHOH is an activated molecule of formaldehyde which undergoes polymerization to hexoses. The photosensitive unit is an adsorption complex or compound of chlorophyll A and hydrated carbon dioxide, and this unit is an oxidation-reduction system which by the absorption of light is converted into chlorophyll B and activated formaldehyde.

It is reasonable to suggest that the Blackman reaction is the reduction of the chlorophyll B to chlorophyll A, possibly by the action of carotin in accordance with the equation

$$C_{55}H_{70}O_6N_4Mg, H_2O + C_{40}H_{56} = C_{55}H_{72}O_5N_4Mg + C_{40}H_{56}O_2.$$
$$\text{Carotin} \qquad\qquad\qquad \text{Xanthophyll}$$
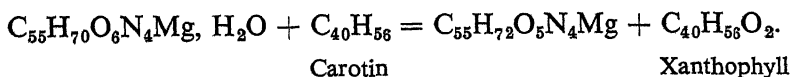
It follows from the foregoing that there are three separate processes which take part in the photosynthetic cycle, namely, the primary light reaction in which the chlorophyll A, CO_2H_2O complex is converted into chlorophyll B and formaldehyde, the Blackman reaction in which

the chlorophyll B is reduced by carotin to chlorophyll A, and the conversion of chlorophyll A into its complex with CO_2H_2O. If a be the concentration of the chlorophyll A, CO_2H_2O complex in the surface when irradiation is commenced, the rate of photosynthesis, when the photostationary state has been established, will be given by

$$r = k_1I (a - x - y) = k_2cxe^{-Q/RT} = k_3py,$$

where I is the intensity of the light, c is the concentration of the carotin which is assumed to be large and sensibly constant, Q is the energy of activation of the Blackman reaction, and p is the pressure of the CO_2.

This expression is based on the assumption that in the dark the concentration of the chlorophyll A, CO_2H_2O complex always returns to the same value a, whatever is the CO_2 concentration, and hence the initial rate of photosynthesis, k_1Ia, will be independent of the CO_2 concentration. Since it has been proved by irradiation with flashing light that the initial rate is a direct function of the external pressure of CO_2, some modification in the assumption must be made. If it be assumed that the rate of formation of the chlorophyll A, CO_2H_2O complex is very great in the case of the chlorophyll A produced in the Blackman reaction and that the attainment of equilibrium between ordinary chlorophyll A and CO_2H_2O is slow, then the above expression of the photostationary state· is simplified to

$$r = k_1I (a - x) = k_2cxe^{-Q/RT},$$

but a is now dependent on the external pressure of CO_2.

If A be the total concentration of chlorophyll A in the surface, both free and in the form of its complex with CO_2H_2O, the concentration a of the complex can be expressed by bA, where b is a function of the external pressure of CO_2, the concentration of water being large and sensibly constant.

Inserting this value of a in the last equation we have

$$r = k_1I (bA - x) = k_2cxe^{-Q/RT} \qquad (1)$$

whence by division by k_1Ix

$$\frac{r}{kIx} = \left[\frac{bA - x}{x}\right] = \frac{k_2c}{k_1I} e^{-Q/RT}$$

and

$$\log \frac{r}{K - r} = \log \frac{k_2c}{k_1I} - Q'/T, \qquad (2)$$

where $K = k_1IbA$ and is the initial rate of photosynthesis and $Q' = Q/2.303R$.

Equations (1) and (2) are in accordance with two of the principal observations since they indicate that the initial rate of photosynthesis is a direct function of the external pressure of CO_2 and that the temperature coefficient is independent of the external pressure of CO_2. It is advisable, before developing the complete formula, to determine whether the linear relation between $\log \dfrac{r}{K-r}$ and $1/T$ indicated by equation (2) conforms with observation.

Now Emerson (1928–29) has made four series of observations of the rate of photosynthesis by *Chlorella* at various temperatures and these are all explained by equation (2). His first series of 10 measurements is expressed by equation $\log \dfrac{r}{130-r} = 22 \cdot 88400 - 6573 \cdot 8/T$. The values of r at various temperatures given by this formula, together with Emerson's observed values, are set forth in Table I.

TABLE I

Temperature	Rate of photosynthesis		Temperature	Rate of Photosynthesis	
°	Calc	Obs	°	Calc	Obs
0	7·79	—	18	86·17	—
1	9·40	—	19	91·17	—
2	11·32	—	20	95·83	—
3	13·24	—	21	100·06	—
4	16·15	13·9	22	103·90	—
5	19·14	—	23	107·33	—
6	22·55	22·7	24	110·37	—
7	26·39	—	25	113·06	—
8	30·67	30·6	26	115·40	115·5
9	35·37	—	27	117·46	—
10	40·46	42·8	28	119·22	—
11	45·89	46·8	29	120·76	—
12	51·58	52·3	30	122·08	—
13	57·45	—	31	123·22	—
14	63·41	64·3	32	124·20	—
15	69·35	—	33	125·03	—
16	75·19	75·3	34	125·75	—
17	80·82	78·5	35	126·36	—

The curve in fig. 1 shows the relation between the calculated values of the rate of photosynthesis and the temperature, the crosses indicating Emerson's measurements. It will be noted that the curve is sigmoid, a fact to which Emerson was the first to draw attention.

Emerson also made three series of measurements with *Chlorella*, with the object of determining the effect of varying the amount of chlorophyll in the alga. Special cultures were prepared and the amount of chloro-phyll present in each was determined. A known volume of the suspension was extracted and the absorptive powers of the solutions of chlorophyll
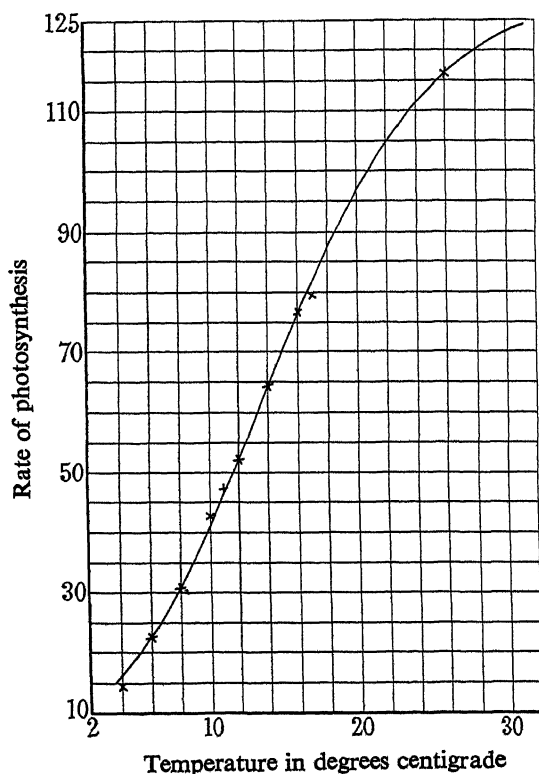
FIG. 1

thus obtained were measured. The extinction coefficients were $\varepsilon = 0 \cdot 101$, $0 \cdot 048$ and $0 \cdot 016$, respectively. His measurements with the three cultures are expressed by the formulæ

$$\varepsilon = 0 \cdot 101 \quad \log \frac{r}{285 - r} = 21 \cdot 891 - 6400/T$$

$$\varepsilon = 0 \cdot 048 \quad \log \frac{r}{210 - r} = 22 \cdot 246 - 6542/T$$

$$\varepsilon = 0 \cdot 016 \quad \log \frac{r}{16 \cdot 3 - r} = 22 \cdot 584 - 6530/T.$$

In Table II are given the calculated and observed values of the rate of

photosynthesis for the three cultures. The values are expressed by the curves in fig. 2, the crosses indicating Emerson's measurements.
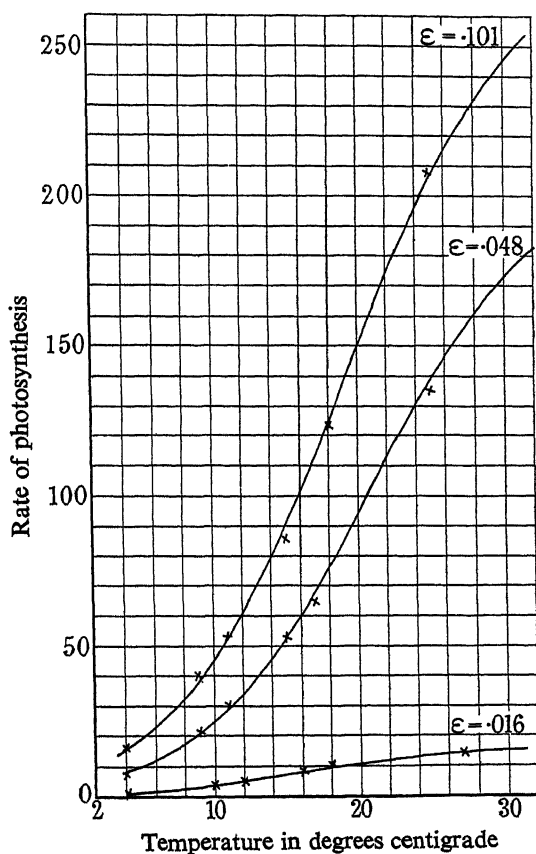
FIG. 2

The values of Q, or the energy of activation of the Blackman reaction, calculated from the four formulæ are:—

Series 1 .... 30,105 calories
$\varepsilon = 0\cdot101$ .... 29,309 ,,
$\varepsilon = 0\cdot048$ .... 29,959 ,,
$\varepsilon = 0\cdot016$ .... 29,905 ,,
Mean .... 29,819·5 ,,

The fact that these values of Q do not diverge much from the mean value in spite of the great differences in the rates of photosynthesis, coupled with the good agreement between the calculated and observed rates, affords evidence of the validity of equation (2).

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TABLE II

Rates of photosynthesis

Temperature	$\varepsilon = 0.101$		$\varepsilon = 0.048$		$\varepsilon = 0.016$	
	Calc	Obs	Calc	Obs	Calc	Obs
0	7·60	—	3·95	—	0·72	—
4	16·41	17·0	8·57	8·1	1·51	—
5	19·63	—	10·30	—	1·80	1·1
9	38·73	40·5	21·04	21·9	3·44	—
10	45·29	—	24·95	—	3·98	4·0
11	52·72	53·4	29·31	30·5	4·57	—
12	61·10	—	34·36	—	5·18	5·0
15	90·57	85·5	53·21	53·2	7·23	—
16	102·10	—	60·67	—	8·03	7·9
17	113·76	—	68·76	64·4	8·77	—
18	125·90	123·9	77·09	—	9·48	10·7
20	150·32	—	95·06	—	10·84	—
25	206·06	208·0	139·00	135·8	13·43	—
27	223·36	—	153·82	—	14·13	14·1
30	243·79	—	171·80	—	14·92	—
35	264·85	—	186·64	—	15·63	—

In view of this satisfactory evidence the influence of the other variables may be considered, but for this purpose it is necessary to derive the complete formula, of which equation (2) is a simplified form. Experimental evidence has been obtained (Baly, 1932) that the primary photosynthetic reaction consists of two consecutive light reactions which are stimulated by light of different wave-lengths. In the living plant these reactions will be represented by:—

(1) $C_{55}H_{72}O_5N_4Mg, CO_2H_2O + h\nu_1 = C_{55}H_{70}O_6N_4MgH_2O, CHOH$

(2) $C_{55}H_{70}O_6N_4MgH_2O, CHOH + h\nu_2 = C_{55}H_{70}O_6N_4MgH_2O$
$$+ CHOH,$$
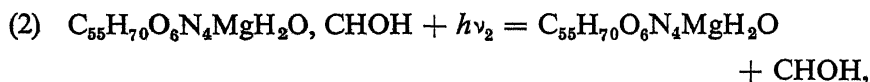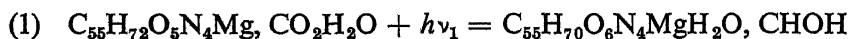
where ν_1 is the frequency of the blue light absorbed by the chlorophyll A, CO_2H_2O complex and ν_2 is the frequency of the red light absorbed by the chlorophyll B, CHOH complex and

$$Nh\nu_1 + Nh\nu_2 = 112,300 \text{ calories.}$$

This mechanism necessitates a different definition of the photostationary state, but as will be seen the only modification thereby caused in the formula is in the term expressing the intensity of the light. If r

once again represents the rate of photosynthesis, the photostationary state will now be expressed by

$$r = k_1 I_1 (bA - p - q) = k_2 I_2 p = k_3 c q e^{-Q/RT}.$$ (3)

By the elimination of p and q we obtain

$$r = \frac{k_1 I_1 b A k_2 I_2 k_3 c e^{-Q/RT}}{k_1 I_1 k_2 I_2 + (k_1 I_1 + k_2 I_2) k_3 c e^{-Q/RT}},$$ (4)

whence

$$\frac{r}{K - r} = \frac{k_1 I_1 + k_2 I_2}{k_1 I_1 k_2 I_2} \cdot k_3 c e^{-Q/RT},$$

where

$$K = \frac{k_1 I_1 k_2 I_2 b A}{k_1 I_1 + k_2 I_2}.$$

But

$$\frac{k_1 I_1 + k_2 I_2}{k_1 I_1 k_2 I_2} = \frac{bA}{K},$$

and hence

$$\log \frac{r}{K - r} = \log \frac{bA k_3 c}{K} - Q'/T.$$ (5)

This equation (5) is identical in form with the simple equation (2), the only difference being that whereas previously the light intensity was defined by I, in the complete equation (5) the light intensity is defined by $I_1 I_2 / (I_1 + I_2)$.

From equation (4) the following relations may be derived:—

$$\frac{bA}{r} = \frac{1}{k_3 c e^{-Q/RT}} + \frac{k_1 I_1 + k_2 I_2}{k_1 I_1 k_2 I_2}$$ (6)

and

$$\frac{I_1 I_2}{r (I_1 + I_2)} = \frac{I_1 I_2}{bA (I_1 + I_2) k_3 c e^{-Q/RT}} + \frac{k_1 I_1 + k_2 I_2}{k_1 k_2 bA (I_1 + I_2)}.$$ (7)

Equation (6) indicates that the rate of photosynthesis is directly proportional to bA when the temperature and light intensities are constant. Since k_1 and k_2 are constants, equation (7) indicates that the ratio of $I_1 I_2 / (I_1 + I_2)$ to the rate of photosynthesis is a linear function of $I_1 I_2 / (I_1 + I_2)$ when the temperature and the ratio I_1/I_2 are constant. Equations (3) to (7) completely define the kinetics of photosynthesis in the living plant when the photostationary state has been established.

VARIATION OF LIGHT INTENSITY

The effect of changing the intensity may be considered first. The problem is two-fold, since the intensity may be varied whilst maintaining

the ratio I_1/I_2 constant or the ratio I_1/I_2 may be varied whilst the total intensity, $I_1 + I_2$, is maintained constant. The former case was investigated by Warburg, who varied the intensity by varying the distance between the light source and calculated the relative intensities from the inverse square law.

In order to determine the effect of reducing the light intensity, $I_1I_2/(I_1 + I_2)$, the equation given above for Emerson's first series of measurements,

$$\log \frac{r}{130 - r} = 22 \cdot 88400 - 6573 \cdot 8/T$$

may be taken. From equation (5) it is obvious that K is directly proportional to $I_1I_2/(I_1 + I_2)$ and that $\log bAk_3c/K$ is inversely proportional to $\log I_1I_2/(I_1 + I_2)$. In Table III are given the values of r at 5°, 10°, 20° and 25° for various relative values of $I_1I_2/(I_1 + I_2)$, together with the temperature coefficients (Q_{10}). The first value of Q_{10} in each case is the square of the coefficient for the range of 5° to 10°.

TABLE III—RATE OF PHOTOSYNTHESIS

Relative value of $\dfrac{I_1I_2}{I_1 + I_2}$	K	5°	Q_{10}	10°	Q_{10}	20°	Q_{10}	25°	30°
1	130	19·14		40·46		95·83		113·06	122·08
			4·47		2·42		1·27		
0·75	97·5	18·25		36·66		76·75		87·65	92·98
			4·04		2·09		1·21		
0·5	65	16·69		30·86		55·16		60·47	62·96
			3·42		1·79		1·12		
0·25	32·5	13·31		20·92		29·84		31·33	31·98
			2·47		1·43		1·05		
0·1	13	8·23		10·64		12·55		12·81	12·92
			1·67		1·18		1·03		
0·05	6·5	5·04		5·85		6·39		6·45	6·48
			1·35		1·10		1·01		
0·025	3·25	2·86		3·08		3·22		3·24	3·24
			1·18		1·05		1·007		
0·01	1·3	1·23		1·27		1·30		1·30	1·30
			1·07		1·02		1·003		

These results are of interest because the effect of reducing the light intensity, whilst maintaining I_1/I_2 constant, is progressively to decrease the temperature coefficient until it approximates to unity. The observations of Blackman and of Warburg, previously referred to, are thus explained.

As already noted, equation (7) indicates a linear relation between the ratio of $I_1I_2/(I_1 + I_2)$ to r and $I_1I_2/(I_1 + I_2)$ when the temperature and I_1/I_2 are constant. Warburg's measurements (Warburg, 1919), of the

rate of photosynthesis with different light intensities (I_1/I_2 and temperature being constant) are expressed by the linear formula

$$I_1I_2/r(I_1 + I_2) = 0 \cdot 016970 + 0 \cdot 0034906 \times I_1I_2/(I_1 + I_2).$$

The curve in fig. 3 shows the relation between $I_1I_2/(I_1 + I_2)$ and r calculated from this formula, the crosses indicating Warburg's observations. As regards his last observation, which does not lie on the curve, Warburg expresses doubt of the value of the light intensity. If the true intensity were $25 \cdot 1$ instead of 45, the observation would lie on the curve.

It may be noted that equation (7) gives a physical explanation of the well-known fact that the efficiency of photosynthesis increases with decreasing light intensity.
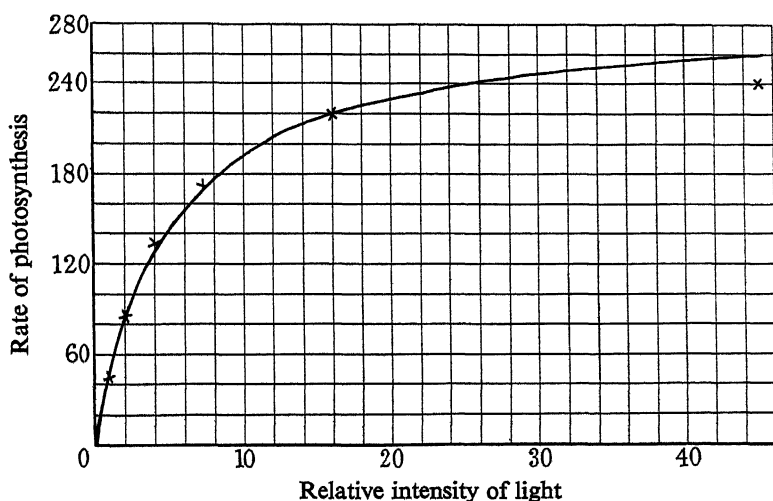
FIG. 3

The second possible variation in the light intensities is a change in the ratio I_1/I_2 whilst maintaining the total intensity of blue and red light $(I_1 + I_2)$ constant. Since $I_1I_2/(I_1 + I_2)$ has a maximum value when $I_1/I_2 = 1$, any change in I_1/I_2 will decrease the rate of photosynthesis even though the total intensity of blue and red light remains constant. This is of considerable interest in view of the fact that various observers have used different light sources which differ in the relative intensities of blue and red light emitted by them. Since in all the usual light sources the ratio I_1/I_2 is equal to or less than unity, the effect of decreasing this ratio may be calculated from the same equation as in the previous case. In Table IV the first two columns contain the values of I_1 and I_2, the sum of these always being 4. In the third column are given the values of

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$I_1 I_2 / (I_1 + I_2)$ and then follow the rates of photosynthesis at 5°, 10°, 20° and 30° and the temperature coefficients (Q_{10}).

As may be seen a progressive reduction in the rate of photosynthesis is caused by a progressive decrease in the ratio I_1/I_2 even though the total intensity of irradiation is kept constant. In order to emphasize

TABLE IV—RATE OF PHOTOSYNTHESIS

I_1	I_2	$\dfrac{I_1 I_2}{I_1 + I_2}$	5°	Q_{10}	10°	Q_{10}	20°	Q_{10}	30°
2	2	1	19·14		40·46		95·83		122·09
				4·47		2·37		1·27	
1·778	2·222	0·9854	19·11		40·30		94·89		120·53
				4·45		2·37		1·27	
1·5	2·5	0·9375	18·96		39·64		91·34		115·16
				4·37		2·30		1·26	
1·333	2·667	0·8868	18·79		38·92		87·59		109·02
				4·29		2·25		1·24	
1·0	3·0	0·7500	18·25		36·66		76·92		92·98
				4·04		2·10		1·21	
0·8	3·2	0·6400	17·68		34·43		67·74		79·89
				3·79		1·97		1·18	
0·667	3·333	0·5556	17·13		32·39		60·28		69·71
				3·58		1·86		1·16	
0·5	3·5	0·4375	16·10		28·90		49·20		55·31
				3·22		1·70		1·12	
0·4	3·6	0·3600	14·99		26·05		41·47		44·71
				3·02		1·59		1·08	
0·25	3·75	0·2344	12·92		20·06		28·12		30·01
				2·41		1·40		1·07	
0·2	3·8	0·1900	11·76		17·39		23·13		24·40
				2·19		1·33		1·05	
0·1	3·9	0·0975	8·10		10·43		12·25		12·60
				1·66		1·17		1·03	
0·05	3·95	0·0494	4·99		5·77		6·31		6·40
				1·34		1·09		1·01	
0·02	3·98	0·0199	2·32		2·48		2·57		2·58
				1·14		1·04		1·006	

this, there are given in Table V the percentage reductions in the rate of photosynthesis with a few values of I_1/I_2 which may be found in the light from various sources in common use.

An interesting confirmation of the effect of varying the relative intensities of the blue and red rays has been given by Dastur and Samant (1933), who found considerable decrease in the amount of carbohydrates formed in the leaves of various plants when daylight, in which the red and blue rays have equal intensity, was substituted by artificial light in which the intensity of the blue rays is materially less than that of the red rays. Recently, Dastur and Mehta* have confirmed this by irradiating plants

* Private communication.

TABLE V

Percentage reduction in rate of photosynthesis

$I_1 : I_2$	5°	10°	20°	30°
1 : 3	4·6	9·4	19·7	23·8
1 : 4	7·6	14·9	29·3	34·6
1 : 5	10·5	20·0	37·1	42·9
1 : 7	15·9	28·6	48·7	55·5
1 : 9	21·7	35·6	56·7	63·4
1 : 15	32·5	50·4	70·7	75·4

with daylight and various artificial lights of equal total intensity. It was found that the smaller the ratio I_1/I_2, the greater was the reduction in the rate of photosynthesis.

It will be at once realized that the decreased photosynthetic efficiency of a light for which I_1/I_2 is small may be counterbalanced by increasing the total intensity until $I_1 I_2/(I_1 + I_2) = 1$. In Table VI are given the factors by which the total intensity must be multiplied to make $I_1 I_2/(I_1 + I_2)$ equal to unity for each of the ratios of I_1 and I_2 given in Table IV. This is of importance in connection with the use by Emerson and Arnold (1932, *b*) of neon light in which the ratio I_1/I_2 is small.

TABLE VI

$I_1 : I_2$	Factor	$I_1 : I_2$	Factor
1 : 1·25	1·0125	1 : 9	2·7778
1 : 1·667	1·0667	1 : 15	4·2667
1 : 2	1·2500	1 : 19	5·2632
1 : 3	1·3333	1 : 39	10·2564
1 : 4	1·5625	1 : 79	20·2532
1 : 5	1·8000	1 : 199	50·2513
1 : 7	2·2857		

It may be seen that as the ratio I_2/I_1 increases the factor approximates more nearly to one-quarter of that ratio.

There is one further case to be considered, namely, the reduction in the intensity of the blue rays by the use of an absorption screen. In order to illustrate this a particular example may be chosen, namely, the investigation of the induction period in photosynthesis by Briggs (1933), who succeeded in proving the existence of the induction period even when the light had been passed through a strong solution of potassium dichromate. Now the molecular extinction coefficient of potassium dichromate* is 2·477 at $\lambda = 4705$ A which lies well within the absorption band of the

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* I am indebted to Dr. R. A. Morton for this measurement.

chlorophyll A, CO_2H_2O complex in the plant. This value of the extinction coefficient indicates that the intensity of the blue rays is reduced to 1/300th after passing through 1 cm of a molar solution or 5 cm of a M/5 solution, the latter being a saturated solution in the case of potassium dichromate. Briggs does not mention the thickness of solution he used but the rate of photosynthesis may be calculated on the assumption that the absorption cell was 2·5 cm in thickness. The ratio of $I_1 : I_2$ in this case is 1 : 150 and the value of $I_1I_2/(I_1 + I_2)$ is 0·013245. Applying this to the same equation as previously the rates of photosynthesis are:—

	10°	20°	25°	30°
No screen	40·46	95·83	113·06	122·08
With screen	1·67	1·71	1·72	1·72

THE TEMPERATURE COEFFICIENT

It will be seen from equations (5), (6) and (7) that the temperature coefficient is a function of $I_1I_2/(I_1 + I_2)$ and the carotin concentration only. It is shown to be independent of the external CO_2 concentration and this was proved to be true by Warburg (1919). Since nothing is known of the carotin concentration, it is not possible to consider this. It may, however, be noted that the effect of decreasing the carotin concentration will be to decrease the velocity of the Blackman reaction, and, as will be shown in discussing the action of poisons, this increases the value of the temperature coefficient. The effect of decreasing $I_1I_2/(I_1+I_2)$ has been shown in Tables III and IV and need not be discussed further, but the fact may be emphasized that two observers using two light sources giving two different ratios of I_1/I_2 will obtain different temperature coefficients for the same temperature range, even though the total energy as measured in calories per second per square decimetre be the same in each case. In Table VII are given the temperature coefficients calculated from the four equations expressing Emerson's observations, together with Warburg's values (1919).

TABLE VII—TEMPERATURE COEFFICIENTS (Q_{10})

| Temperature range °	Series 1	$\varepsilon = 0·101$	$\varepsilon = 0·048$	$\varepsilon = 0·016$	Warburg
0–10	5·65	5·96	6·31	5·53	—
5–10	4·47	5·32	5·86	4·90	5 to 4·3
10–20	2·42	3·32	3·81	2·72	2·1
20–30	1·27	1·62	1·80	1·37	1·6
25–35	1·12	1·09	1·34	1·16	—

VARIATION IN THE CARBON DIOXIDE CONCENTRATION

Equation (6) indicates that when the temperature and $I_1 I_2/(I_1 + I_2)$ are constant the rate of photosynthesis is directly proportional to bA, that is to the fraction of the total chlorophyll A molecules which has adsorbed hydrated CO_2. Now the fraction b is a function of the external CO_2 concentration and by the Langmuir adsorption formula we have

$$bA = \frac{sP}{1 + sP} ,$$

where P is the external CO_2 concentration and s is a constant, the concentration of H_2O being assumed to be large and sensibly constant. Substituting this value of b in equation (6) it may be seen that

$$P/r = B + CP,$$

Concentration of CO_2 in moles $\times 10^{-6}$ per litre
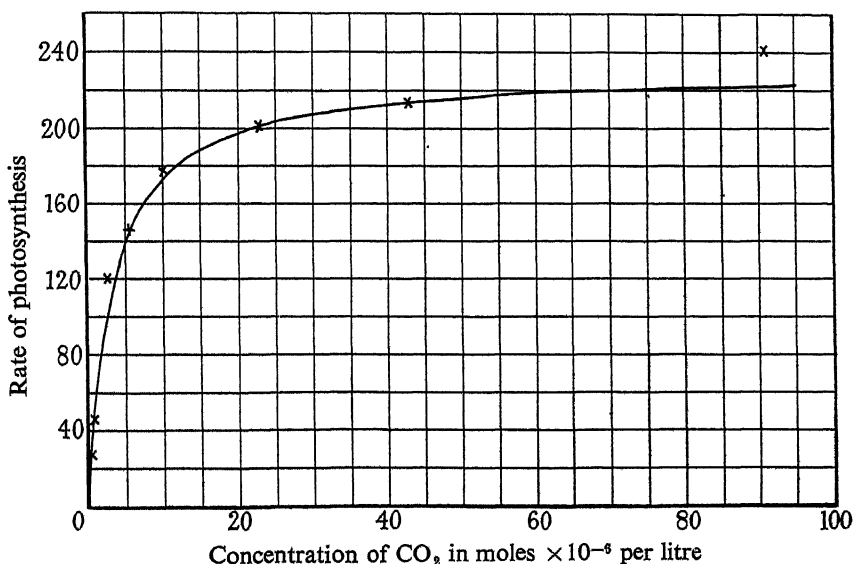
FIG. 4

where B and C are constants when both $I_1 I_2/(I_1 + I_2)$ and the temperature are constant. This formula indicates that the ratio of the external CO_2 concentration to the rate of photosynthesis should be linearly proportional to the external CO_2 concentration.

The P, r curve in fig. 4 is that given by the linear equation

$$P/r = 0.013400 + 0.0043224\ P$$

and Warburg's (1919) measurements are represented by the crosses.

As will be seen, these lie sufficiently well with respect to the curve with the exception of the last one when P $= 91 \times 10^{-3}$ mole of CO_2 per litre. This deviation from the formula at high concentration of CO_2 is of some interest, especially since Warburg pointed out that at the three highest concentrations of CO_2 the rate of photosynthesis is linear with the concentration. Whilst it is possible that the calculation of the CO_2 concentrations from the activity coefficients instead of the dissociation constants might give different values at the higher concentrations and therefore a new linear formula, it would seem more probable that the deviation is due to a more fundamental cause.

Now the external CO_2 concentration under which photosynthesis normally takes place in plants is that present in the atmosphere, namely, from 1·34 to 1·79 $\times 10^{-5}$ mole per litre. Furthermore, it is obvious that, as the external CO_2 concentration is progressively increased, the fraction b of the molecules of chlorophyll A in the surface which has adsorbed hydrated CO_2 must increase until b is equal to unity, when the whole of the surface molecules are coated with CO_2H_2O. The question then arises as to what will happen if the external CO_2 concentration is still further increased. Since Emerson and Arnold (1932, c) have proved that the number of chlorophyll molecules which actually function in photosynthesis is only a small fraction of the total number present, it is possible that when the external CO_2 concentration is greater than that which gives $b = 1$, interpenetration of the space lattice of the chlorophyll by the hydrated CO_2 begins, with the result that the effective surface will be increased and the rate of photosynthesis enhanced beyond that given by the linear formula.

The highest concentration of CO_2 used by Warburg in the series of measurements under discussion was 9·1 $\times 10^{-5}$ mole per litre, which is from 5 to 6·8 times that normally present in the atmosphere. If this concentration is sufficiently great to cause interpenetration of the chlorophyll space lattice, then the enhanced rate of photosynthesis at that concentration will find an explanation.

IRRADIATION WITH FLASHING LIGHT

It is well known that, all other factors being constant, the efficiency of photosynthesis is materially increased by intermittent illumination. It was shown by Warburg that the increase in the rate of photosynthesis is the greater the shorter is the period of each flash, and Emerson and Arnold proved that the greatest increase is obtained when the period of

each flash is very short and the dark periods are sufficiently long for the Blackman reaction to complete itself.

For the sake of convenience the equation expressing the rate of photosynthesis when the photostationary state has been established may be re-stated

$$r = k_1 I \, (bA - p - q) = k_2 I_2 p = k_3 c q e^{-Q/RT}$$

and it follows from the equation that when the irradiation is stopped and the Blackman reaction completes itself, the concentration of the chlorophyll A, CO_2H_2O complex will increase from $bA - p - q$ to $bA - p$, whilst the concentration of the chlorophyll B, CHOH complex will remain unchanged. If continuous irradiation with the same light be then started, the initial velocity of the first reaction will be $k_1 I \, (bA - p)$ and the initial velocity of the second reaction will be $k_2 I_2 p$. These velocities, however, are not equal and consequently there will be an interval of time before the photostationary state is re-established, that is to say there must be a period of induction. On the other hand if, after a period of darkness, irradiation be carried out for very short periods, each period being separated by a sufficiently long dark period for the Blackman reaction to complete itself, a new condition will slowly be established. The shorter the period of each light flash, the more nearly will this condition approximate to the equation

$$r = k_1 I_1 \, (bA - p') = k_2 I_2 p'$$

whence

$$r = k_1 I_1 bA \left(1 - \frac{k_1 I_1}{k_1 I_1 + k_2 I_2} \right).$$

This expression gives the maximum rate of photosynthesis in flashing light when the period of each flash is reduced to a minimum. It indicates that the rate will be greater, the greater is the total intensity of light, and that with very great intensity the rate will approximate to $k_1 I_1 bA$. It follows that $k_1 I_1 bA$ is the maximum possible rate of photosynthesis in flashing light.

Now it is not possible to determine the value of $k_1 I_1 bA$ from the equations for the photostationary state, but it may be seen from these equations that $K = k_1 I_1 bA \times \dfrac{k_2 I_2}{k_1 I_1 + k_2 I_2}$, and, since $k_2 I_2 / (k_1 I_1 + k_2 I_1)$ must be less than unity, it follows that $k_1 I bA$ must be greater than K. In Table III were given the rates of photosynthesis at five different temperatures, together with the relevant values of K, when the relative value of $I_1 I_2 / (I_1 + I_2)$ is reduced in the formula for Emerson's first series, $\log r / (130 - r) = 22 \cdot 88400 - 6573 \cdot 8/T$.

A measure of the change in the rate of photosynthesis when flashing light is substituted for continuous irradiation is given by the difference between the observed rate and the relevant value of K. The observed change will be greater than this, but the smaller value will be sufficient for the present purpose.

It will be seen that the difference between the value of K and the rate in continuous light is smaller, the smaller is the intensity of the light and the higher is the temperature. The observations of Warburg (1919) and of Emerson and Arnold (1932, b) have established these relations. The latter also stated that the rate in flashing light is linearly proportional to the total intensity and this suggests that the rate was very little different from the theoretical value $k_1 I_b bA$, since this is linearly proportional to the total intensity when I_1/I_2 is constant.

The increases in the rate of photosynthesis indicated in Table III are much smaller than those observed by Warburg and by Emerson and Arnold. This is due to the fact that Emerson's observations expressed by the formula given above were made with relatively low light intensity. This fact may be proved by increasing the value of $I_1 I_2/(I_1 + I_2)$ in the formula, when very great increases in the rate of photosynthesis are obtained. As may be seen from fig. 3, this would not be true if the intensity were already very large, since in this case an increase in the intensity does not increase the rate to any marked extent. In Table VIII is shown the effect of increasing $I_1 I_2/(I_1 + I_2)$ together with the percentage increase in the rate when by flashing light the rate is increased to the relevant value of K. The percentage increases are given because Warburg and Emerson and Arnold expressed their results in this way. It must be remembered that the actual increases observed when changing from continuous to flashing light will be greater than those in the Table.

TABLE VIII

$\dfrac{I_1 I_2}{I_1 + I_2}$	K	Rate of photosynthesis in continuous light				Percentage increase in rate in flashing light			
		10°	20°	25°	30°	10°	20°	25°	30°
1	130	40·46	95·85	113·06	122·08	221·3	35·6	15·0	6·5
5	650	53·87	233·57	371·56	490·88	1106·5	178·3	74·9	32·4
10	1300	56·20	284·71	520·26	788·74	2213·0	355·8	149·9	64·8
50	6500	58·22	345·19	765·30	1523·70	11065·0	1783·0	749·3	324·1

As may be seen, the system becomes almost " saturated " with light at 10° when $I_1 I_2/(I_1 + I_2)$ is increased five times. At the higher temperatures the system is not saturated even when $I_1 I_2/(I_1 + I_2)$ is increased 50 times.

THE EFFECT OF POISONS

A poison may affect the rate of photosynthesis in two ways. It may either decrease the velocity of the Blackman reaction or it may decrease the velocity of the primary photosynthetic reaction by being preferentially adsorbed by the chlorophyll A and thus preventing the latter from adsorbing or combining with hydrated CO_2. Since the effect is different in the two cases, they may be separately discussed and as previously the formula

$$\log \frac{r}{130 - r} = 22 \cdot 88400 - 6573 \cdot 8/T$$

may be used.

The effect of reducing k_3c in the formula, that is to say the velocity of the Blackman reaction, is shown for four temperatures in Table IX.

TABLE IX

Relative value of k_3c	Rate of photosynthesis						Percentage reduction in rate			
	10°	Q_{10}	20°	Q_{10}	25°	30°	10°	20°	25°	30°
1·0	40·46		95·83		113·06	122·08				
		2·42		1·27						
0·5	23·96		75·88		100·02	115·11	40·78	20·82	11·53	5·71
		3·17		1·52						
0·2	10·77		46·71		71·31	98·08	73·38	51·26	35·89	19·66
		4·33		2·10						
0·13	7·21		34·73		60·38	86·75	82·18	63·76	46·60	28·94
		4·81		2·50						
0·10	5·60		28·47		52·03	78·87	86·16	70·29	56·52	35·39
		5·07		2·77						
0·05	2·87		15·97		32·52	56·61	90·90	83·34	71·24	53·63
		5·57		3·54						
0·02	1·16		6·90		13·31	30·55	97·13	92·70	88·02	74·98
		5·92		4·43						
0·01	0·58		3·55		8·13	17·35	98·57	96·30	92·81	85·78
		6·06		4·90						
0·001	0·06		0·36		0·86	1·98	99·85	99·63	99·24	98·38
		6·19		5·44						

As may be seen the effect of progressively poisoning the Blackman reaction is very materially to reduce the rate of photosynthesis and to increase the temperature coefficient.

The most interesting result is the effect of decreasing the light intensity with a constant amount of poisoning. This is shown in Table X for three amounts of poisoning and four intensities of light.

The figures given in Table X show that the effect of a given amount of poisoning is progressively decreased when the light intensity is reduced,

this effect being greatest at the higher temperatures. This effect was observed by Warburg (1919 and 1920) when *Chlorella* was poisoned by HCN. He found that with a concentration of $3\cdot8 \times 10^{-5}$ moles HCN per litre the effect of the poisoning was reduced from 48% to 1% when the light intensity was reduced to approximately 1/50. This reduction is very similar to that shown in Table X when the relative value of $k_3 c$ is reduced to $0\cdot13$; the reduction in the rate of photosynthesis at 25° when $I_1 I_2/(I_1 + I_2) = 1$ is 46·6% and when $I_1 I_2/(I_1 + I_2) = 0\cdot02$ is 1·93%.

TABLE X

$\dfrac{I_1 I_2}{I_1 + I_2}$	$k_3 c$	Rate of photosynthesis				Percentage reduction in rate			
		10°	20°	25°	30°	10°	20°	25°	30°
1·00	1·0	40·46	95·83	113·06	122·08				
	0·5	23·96	75·88	100·02	115·11	40·78	20·82	11·53	5·71
	0·13	7·21	34·73	60·38	86·75	82·18	63·76	46·60	28·90
	0·01	0·58	3·55	8·13	17·35	98·57	96·30	92·81	85·78
0·05	1·0	5·85	6·39	6·45	6·48				
	0·5	5·05	6·28	6·40	6·46	13·87	1·72	0·78	0·34
	0·13	3·51	5·72	6·15	6·34	40·00	10·49	4·65	2·16
	0·01	0·54	2·34	3·72	4·91	90·75	63·38	42·33	24·15
0·02	1·0	2·49	2·58	2·59	2·60				
	0·5	2·39	2·56	2·58	2·59	4·02	0·78	0·39	0·32
	0·13	1·94	2·15	2·54	2·57	22·09	16·67	1·93	1·15
	0·01	0·48	1·52	2·00	2·30	80·72	41·09	22·78	11·54
0·01	1·0	1·27	1·30	1·30	1·30				
	0·5	1·25	1·29	1·30	1·30	1·57	0·77	0	0
	0·13	1·11	1·27	1·28	1·30	14·56	2·31	1·54	0
	0·01	0·40	0·96	1·13	1·22	68·50	26·15	13·08	5·74

It is satisfactory to note that by reducing the veloeity of the Blackman reaction alone the effects observed by Warburg are quantitatively explained. The primary photosynthetic mechanism is not affected, therefore, as Warburg proved by his observations.

In the second case the effect of the poison is to decrease the velocity of the primary photosynthetic reaction owing to the chlorophyll A adsorbing the poison preferentially to the hydrated carbon dioxide. If n is the fraction of the chlorophyll molecules in unit area which have adsorbed the narcotic, then by Langmuir's formula

$$n = \frac{s\,N}{1 + s\,N},$$

where N is the external concentration of the narcotic and s is a constant depending on the nature of the narcotic. The number of unpoisoned molecules of chlorophyll A in unit area is $A(1 - n)$ and hence the velocity of photosynthesis will be a function of $bA(1 - n)$.

Since

$$1 - n = \frac{1}{1 + sN},$$

and since the rate of photosynthesis is directly proportional to bA, it follows that

$$\frac{1}{r} = C + DN,$$

where C and D are constants. The reciprocal of the rate of photosynthesis should, therefore, be a linear function of the external concentration of the narcotic.

Warburg's (1920) observations with phenyl urethane are expressed with reasonable accuracy by the linear equation

$$\frac{1}{r} = 0 \cdot 011000 + 0 \cdot 003114 \, N.$$

Assuming that in the absence of narcotic the rate of photosynthesis is 100, the calculated and observed values are given in Table XI.

TABLE XI—POISONING BY PHENYLURETHANE

N × 10⁴ moles per litre	1·2	2·4	4·8	9	18	36
1/r	0·01473	0·01847	0·02593	0·03900	0·08118	0·12300
r	67·9	54·1	38·6	25·6	12·3	8·1
Reduction in r..	32·1	45·9	61·4	74·4	87·7	91·9
Obs. (Warburg)	37	46	65	74	86	95

As already stated, the efficiency of a narcotic is measured by the adsorption constant s in the Langmuir formula. Warburg gives the concentrations in millimoles per litre for six substituted urethanes which reduce the rate of photosynthesis by 50%. From these the relative values of the adsorption constant s can readily be obtained, since they are inversely proportional to the equivalent concentrations. These values are given in Table XII.

In conclusion it may be claimed that the formulæ derived for the rate of photosynthesis during the photostationary state give a satisfactory explanation of the observations that have been made of (1) the effect of

changing the four variables, temperature, CO_2 concentration, total light intensity, and the relative intensities of blue and red light, (2) the effect of irradiation by flashing light, and (3) the effect of poisons, in the case of the uni-cellular alga *Chlorella*.

TABLE XII

Narcotic	Concentration in millimoles per litre which reduces r by 50%	Relative values of adsorption constants
Methylurethane	400	1·00
Ethylurethane	220	1·82
Propylurethane	50	8·00
*iso*Butylurethane	17	23·53
*iso*Amylurethane..........	12	33·33
Phenylurethane	0·5	80·00

I have to express my thanks to Dr. R. O. Griffith and Dr. A. McKeown for their valuable assistance in defining the conditions governing the photostationary state and also to Dr. L. B. Morgan for his interest in the problem.

SUMMARY

It has been proved that the primary process in photosynthesis is an oxidation-reduction reaction. Expressing this in the simplest terms of chlorophyll a complex of chlorophyll A and hydrated CO_2 is converted by the action of light into chlorophyll B and activated formaldehyde which at once undergoes polymerization into hexoses. The chlorophyll B thereby produced undergoes the dark or Blackman reaction and is reduced to chlorophyll A. There are, therefore, three separate processes involved in the photosynthetic cycle, namely, the primary photosynthetic reaction, the Blackman reaction, and the formation of the photosensitive complex of chlorophyll A and hydrated CO_2.

Since the Blackman reaction regenerates chlorophyll A from the chlorophyll B produced in the primary reaction, a photostationary state will be established which is determined by the intensity of irradiation, the temperature and the external concentration of hydrated CO_2. In this communication the equation for the photostationary state is derived and shown to express correctly the variation in the rate of photosynthesis with temperature.

In view of the evidence that the primary photosynthetic process consists of two consecutive light reactions, one promoted by blue light and the other by red light, the equations for the photostationary state are modified by the substitution of the term $I_1 I_2/(I_1 + I_2)$ in which I_1 and I_2 are the

intensities of the blue and red rays, respectively. The equations give a satisfactory explanation of (1) the effect of changing the four variables, temperature, CO_2 concentration, total light intensity, and the relative intensities of blue and red light, (2) the effect of irradiation by flashing light, and (3) the effect of poisons, in the case of the uni-cellular alga *Chlorella*.

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On the Ampholytic Nature of Phospholipins

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INTRODUCTION

Of the N-containing phospholipins (Levene and Rolf, 1921; McLean and McLean, 1927; Thierfelder and Klenk, 1930; Magistris, 1931, Robison, 1932; Klenk, 1933) at present three groups have been fairly accurately defined and isolated in an almost pure state:—

$$O = P \begin{cases} -\text{O . diglyceride} \\ -\text{O . choline} \\ -\text{OH} \end{cases} \quad O = P \begin{cases} -\text{O . diglyceride} \\ -\text{O . colamine} \\ -\text{OH} \end{cases} \quad O = P \begin{cases} -\text{O . (fatty acid) sphingosine} \\ -\text{O . choline} \\ -\text{OH} \end{cases}$$

I	II	III
Lecithin	Cephalin	Sphingomyelin
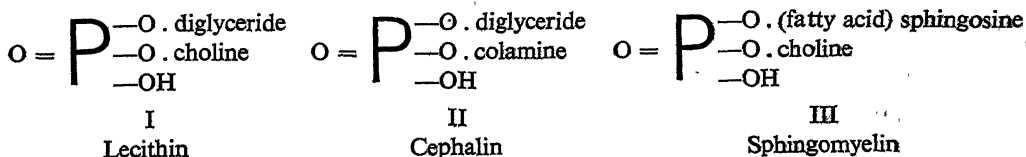
These compounds are diesters of phosphoric acid. One acid group is esterified with an alcoholic base which may be either colamine (amino-ethylalcohol $NH_2 . CH_2 . CH_2 . OH$) or the corresponding quaternary ammonium base choline ($OHN(CH_3)_3 . CH_2 . CH_2 . OH$). The second acid group is linked in the sphingomyelin with sphingosine, the amino group of which is substituted by a fatty acid radical, or in lecithin and cephalin with a diglyceride. The analogous compounds lysolecithin and lysocephalin contain a monoglyceride instead of a diglyceride.

The structure of the known phospholipins have as a common characteristic the presence of two ionogenic groups, namely, the free phosphoric group and the free basic group of either aminoethylalcohol or choline. In spite of a great deal of work on the chemistry of phospholipins, little attention has been paid to the behaviour of the polar groups. This is probably due to the fact that there are no adequate methods available for studying their character. A titration, determining the acid—binding and base—binding power of the phospholipins, would provide such a method. Grün and Limpächer (1926, 1927) made titrations of phospholipins and their method was applied by Rudy and Page (1930). These workers found that, using as indicator phenolphthalein, cephalin in benzene—alcoholic solution titrates as a monobasic acid, while lecithin in the same condition is neutral to phenolphthalein. According to older conceptions they explain this finding by assuming that in the case of cephalin—unlike lecithin—the phosphoric radical is dominant on account of the weak basic nature of aminoethylalcohol.

It was our purpose to develop a titration method with which it would be possible to detect not only the base binding, but also the acid binding properties of the phospholipins.

THEORETICAL

The phenomena occurring during titrations in non-aqueous solvents were first explained by the fundamental theoretical work of Brönsted (1923, 1926, 1928, 1929, 1930, 1934). The usual conception that an acid is defined by the dissociation of H^+-ions, and a base by the dissociation of OH^--ions loses its meaning in solvents in which no dissociation of the solute takes place. Brönsted defines an acid as a substance which is capable of giving up a proton (H^+-ion) and a base as a substance which is capable of taking up a proton.

$$A \;\rightleftharpoons\; B \;+\; H^+$$
$$\text{(acid)} \quad \text{(base)} \quad \text{(proton)}$$
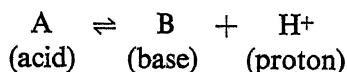
The proton can be given up by an acid only when a substance is present which is capable of taking up the proton, since H^+-ions are not capable of existence in a free state.

The acidity of a solution is defined by the equation

$$\mu = kT \, ln \, a_{H^+}$$

where μ is the " acidity potential," and a_{H^+} the H^+-ion activity. The acidity constant K_{acid} of a particular acid is given by the expression

$$K_{acid} = a_{H^+} \frac{c_B}{c_A}$$

where c_A and c_B denote the stoichiometric concentrations of the acid and base involved, while a_{H^+} denotes not the concentration of the H^+-ions, but their thermodynamic activity. The acidity is obviously considered in the same way as a redox potential where also the assumption of free electrons is unnecessary. In a similar way the basicity constant is defined:

$$\frac{1}{a_{H^+}} \cdot \frac{c_A}{c_B} = K_{bas}.$$

It appears from this that

$$K_{acid} \cdot K_{bas} = 1.$$

When two acid base systems are present in a solution (" double acid-base system " or " double buffer system ") a proton exchange must occur

$$A_1 + B_2 \rightleftharpoons A_2 + B_1$$
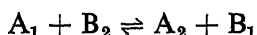
until the condition for the equilibrium

$$\frac{K_{acid_1}}{K_{acid_2}} = \frac{c_{B_1} \cdot c_{A_2}}{c_{A_1} \cdot c_{B_2}} \tag{1}$$

is fulfilled.

According to the capability of a solvent to take up or give up protons one has to distinguish between:—

1—aprotic solvents (benzene, toluene, paraffin, chloroform; not giving up or taking up protons);
2—acidic solvents (acetic acid, sulphuric acid, hydrogen-fluoride; protons given up only);
3—basic solvents (liquid ammonia, aniline; protons taken up only);
4—amphiprotic solvents (water, alcohol; both taking up and giving up protons).

Actual dissociation of an acid is only possible in basic or amphiprotic solvents and is a special case of a process occurring between two acid-base systems; e.g., for the dissociation of an acid in water we have the relations:

$$A + H_2O \rightleftharpoons B + H_3O^+.$$
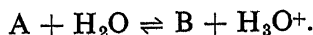
The water forms a buffer system, the acid-base strength of which, according to its ampholytic nature, is described by two pairs of constants

(1)
$$H_3O^+ \rightleftharpoons H_2O + H^+$$

$$K_{W\,acid_1} = a_{H^+} \cdot \frac{c_{H_2O}}{c_{H_3O^+}} = \frac{1}{K_{W\,bas_1}}$$

(2)
$$H_2O \rightleftharpoons OH^- + H^+$$

$$K_{W\,acid_2} = a_{H^+} \frac{c_{OH^-}}{c_{H_2O}} = \frac{1}{K_{W\,bas_2}}.$$

The dissociation equilibrium of an acid is determined by the relation

$$\frac{K_{acid}}{K_{W\,acid_1}} = \frac{c_B \cdot c_{H_3O^+}}{c_A \cdot c_{H_2O}}, \tag{2}$$

where $c_B/c_A \cdot c_{H_2O+}$ is the dissociation constant K_C in the old sense. Therefore, except for a constant factor, the dissociation constant for an acid equals the product of its acidity constant and the basicity constant of the solvent used:

$$K_C = K_{acid} \cdot K_{W\,bas_1}.$$

Analogous relations are true for bases, which can dissociate only in acidic or amphiprotic solvents. From these considerations it appears that in a solvent allowing dissociation, every acid of strength above a certain limit must dissociate almost completely with formation of "lyonium" ions (that is, the acid form of the solvent).

Thus, for example, in water all acids above a certain strength must form almost completely the oxonium-ion H_3O^+, e.g.,

$$HCl + H_2O \rightleftharpoons H_3O^+ + Cl^-,$$
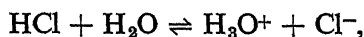
and the H_3O^+-ion is the new formed acid. It appears that, contrary to the classical conception, the dissociation does not determine the strength of an acid, since, in fact, a weaker acid is formed by dissociation from the stronger one. This formation of a weaker acid from a solution of a stronger acid in an amphiprotic solvent (such as water) produces a "levelling down" effect, which provides an explanation of the apparent equality of strength of all such solutions of strong acids. In aprotic

solvents that levelling down does not exist. It was found (Goldschmidt, 1907, 1926; Hantsch *et al.*, 1923, 1927, 1929) that the strength of acids decreases in the order

$$HClO_4 > HBr > HCl.$$

While on the basis of these considerations the basicity or acidity of the solvent is an important factor in the dissociation of acids, still another factor has to be considered, namely, the dielectric constant of the solvent, which influences the strength of acids more directly. According to the Nernst-Thomson rule, the smaller the dielectric constant, the bigger is the attraction of opposite and the repulsion of like charges. This manifests itself in a displacement of the acidity constant of an acid when dissolved in two media of different dielectric constants. Here the different types of acids behave differently. Cationic acids change their K_{acid} only slightly, because with a proton exchange there is no change in charges:

$$A^+ \rightleftharpoons B^{(0)} + H^+.$$
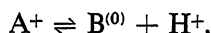
Uncharged acids, however, develop a smaller K_{acid} in media with a smaller dielectric constant

$$A^{(0)} \rightleftharpoons B^{(-)} + H^+$$
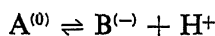
(Michaelis and Mizutani, 1925; Mizutani, 1925).

EXPERIMENTAL

Solvent—In the following titrations a mixture containing 19 volumes benzene and 1 volume absolute alcohol was used as solvent. The benzene was chosen because of its ability to dissolve the phospholipins and because of its low dielectric constant. The addition of the alcohol was necessary to keep in solution all substances used in the titration: phospholipins, titrant, and indicator. The dielectric constant of this mixture can be calculated approximately from the values of both the constituents according to the simple mixing rule; it is about $3 \cdot 5$ (benzene $2 \cdot 2$, alcohol 27) the medium therefore must be described as an amphiprotic solvent with a very low dielectric constant.

Titrant—The same solvent was used for the titrant, which contained a certain amount of acid and base. Sodium ethylate was used as base since the ethylate ion is the strongest possible base in this solvent. Metallic sodium was dissolved in absolute alcohol and made up to $0 \cdot 4$ N: one part of this solution was mixed with 19 parts benzene. Thus the solution was a $0 \cdot 02$ N alkali solution.

The acid titrants were made either by passing gaseous HCl through alcohol until it was $0\cdot4$ N, or by dissolving 10 N $HClO_4$ (65%, sp. gr. $1\cdot6$: higher concentrations were not used because of the danger of explosions) in 25 parts absolute alcohol. The solutions were further diluted with benzene 1 : 20. The solutions were standardized with standard HCl or NaOH solutions in alcohol with bromthymol blue as indicator. 25 cc of the benzene-alcohol mixture were titrated against different indicators to compare the two acid titrants. The indicators were made up in $0\cdot1\%$ alcoholic solution, 5 drops of which were used for every titration. Table I shows the results. The two values for each indicator correspond to equal colour intensity.

TABLE I

Indicator	$HClO_4$	HCl
Naphthyl red........................	1 drop	1 drop
Methyl red..........................	$\begin{cases} 0\cdot02 \\ 0\cdot06 \\ 0\cdot12 \end{cases}$	$\begin{matrix} 0\cdot27 \\ 0\cdot60 \\ 1\cdot60 \end{matrix}$
Dimethylamino-azobenzene ...	$\begin{cases} 0\cdot16 \\ 0\cdot2 \end{cases}$	$\begin{matrix} 0\cdot6 \\ 1\cdot2 \end{matrix}$
Pentamethoxy - triphenylcar - binol (Lund)*	$\begin{cases} 0\cdot2 \\ 0\cdot3 \end{cases}$	$\begin{matrix} 5\cdot0 \\ \infty \end{matrix}$

* We are much indebted to Professor H. Lund for the sample of pentamethoxy-triphenylcarbinol.

According to the theory outlined above all strong acids should be equally effective owing to the formation of the ethoxonium ion, but these results indicate that $HClO_4$ is much stronger than HCl, for with each indicator, it required a greater amount of HCl than of $HClO_4$ to produce the same colour intensity.

It might be expected that the ratio of the acid strengths, colorimetrically determined with different indicators would be constant. This is not so. The fact must be considered that in solutions of low dielectric constant the ionic strength has a much greater influence than in water (Debye and Hückel, 1923; Hückel, 1925; Williams, 1931. The " salt error," based on this fact, and the tendency to association is different for every indicator. However, in spite of individual peculiarities, the indicators still show clearly the general behaviour.

The behaviour of the HCl is obviously conditioned by the decrease of its acidity constant in a solvent of very low dielectric constant. The dissociation of HCl is determined by:

$$K_{diss\ HCl} = K_{acid\ HCl} \cdot K_{bas\ C_2H_5OH} = \frac{K_{acid\ HCl}}{K_{acid\ C_2H_5OH_2}^+}$$

where $K_{acid \, HCl}$, as an acidity constant of an uncharged acid, is dependent to a high degree upon the dielectric constant, while $K_{acid \, C_2H_5OH_2^+}$, as the acidity constant of a cationic acid, is much less influenced. Even though HCl is strong enough for complete dissociation in pure alcohol (this has not been proved definitely, compare Schreiner (1924)) it cannot dissociate completely in a solvent with a very low dielectric constant. The stronger $HClO_4$ was chosen for use in the following titrations.

Indicators—The suitable indicator has to fulfil the following conditions:—

1—it must be readily soluble in the medium used;
2—the colour change should be as far as possible on the acid side for the acid titration, and for the alkaline titration it should be as far as possible on the alkaline side;
3—the colour change should easily occur with N/50 solutions so that large reading in the blank titration can be avoided.

a—*Indicators on the Alkaline Side*—Indicators of acid nature are suitable because their range of colour change is shifted to the alkaline side when brought into media with lower dielectric constants. A 0·1% solution of phenolphtalein in the benzene-alcohol mixture (19: 1) was used as indicator. Of other indicators tested, bromthymol blue was rejected as not alkaline enough, thymolphtalein as insufficiently soluble in benzene, and trinitrobenzene, since it becomes pale in alkaline solution and loses its indicator properties (Kolthoff, 1932).

b—*Indicators on the Acid Side*—Only indicators of basic nature with colour change on the acid side were tested, because, in these, the range of colour change is not influenced very much by the dielectric constant. Of the indicators tested methyl violet, metanil yellow, and tropaeolin 00 were found too acid, methyl red and α-naphtyl red too alkaline. Pentamethoxytriphenylcarbinol (Lund) and dimethyl yellow are both suitable; the latter, also in 0·1% solution, was used because it has a good colour intensity and the colour can be observed and compared even if the phosphatide which is titrated is itself coloured.

Concentration of the Phospholipins—All titrations were carried out in a volume of 25 cc. It is essential to keep the concentration of the phospholipins low because the salt effect in solution of low dielectric constant is very big. In addition the dielectric constant is increased in the presence of dipoles and the possibly repressed acid group might dissociate again. In fact, the results of the titrations were unreliable if

the concentration was bigger than 0·002 molar. The addition of electrolytes was also very disturbing. The concentration of 0·002 molar is, however, completely sufficient as 1/20 millimol in 25 cc can be easily titrated with N/50 solutions.

PREPARATIONS

Lecithin—Lecithin was prepared by the method of Levene and Rolf (1927). The treatment with diluted acetic acid, proposed by the authors for removal of traces of ammonia, involves the danger of partial hydrolysis and the removal of the last traces of acetic acid is very difficult (compare Maltaner (1930)). Therefore this refinement was omitted; the preparations gave good analytical data after once repeated precipitation as cadmium salt. The preparations were completely white after removal of the cadmium chloride. Analysis: $N = 1·8\%$; $P = 3·96\%$.

Cephalin—Cephalin was prepared from ox brains following the usual methods. The brains after drying with acetone were exhaustively extracted with ether, the etherial extract was condensed under CO_2 and kept in the ice chest to precipitate the " white material." After filtering off the precipitate, the extract was precipitated with alcohol and the same operations repeated three times. In spite of all precautions the preparations were brownish. Analysis: $N = 1·9\%$; $NH_2—N$ (van Slyke) $= 1·8\%$; $P = 3·6\%$.

Lysolecithin—The preparation was made according to the directions of Levene, Rolf, and Simms (1924) from egg yolks. The crude product was recrystallized three times from benzene and once from absolute alcohol. Lysocephalin was not separated. Analysis: $P = 6·0\%$; $N = 3·0\%$; $NH_2 — N$ (van Slyke) $= 0·6\%$. The preparation contained 20% lysocephalin.

Sphingomyelin—The preparations were made according to the directions of Levene (1914, 1916) and of Merz (1930). The preparation made according to Levene after many recrystallizations from pyridin-chloroform still showed low N-values. In addition all preparations in benzene were acid in phenolphthalein (compare Frankel, Bialschowsky, and Thannhauser, 1933, footnote). After further purifications by the cadmium compound much less acid preparations, giving better analytical data, were obtained. A trace of acid impurity was still found after the preparation had undergone cadmium purification three times. Analysis: $N = 3·4\%$; $P = 3·995\%$.* Optical rotation: 0·4016 gm dissolved in

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* The microanalyses were carried out in the Biochemical Institute, Cambridge, by Mr. A. Colwell, to whom we wish to express our best thanks.

4·3 cc chloroform gave in a 2 dm tube a rotation of $+ 1·10°$, from this an $[\alpha]_D^{20} = + 6·06°$ is calculated. This value is in fair accordance with the value given by Merz. If the lipide mixture is fractionated with glacial acetic acid instead of chloroform-methylalcohol, a rapid separation of the cerebrosides from sphingomyelin can be made (Merz). Protagon is dissolved in 5 parts glacial acetic acid at 70°, and the precipitate forming after 1 hour at room temperature is filtered off (it consists mostly of galactolipins). A further precipitate separates from the filtrate after 12 hours (consisting mostly of phrenosin mixed with sphingomyelin) this is filtered off, and redissolved in glacial acetic acid. The precipitate which forms on standing having been filtered off, the two filtrates are mixed and evaporated to a small volume *in vacuo*, and precipitated with acetone. The precipitate is dissolved in a ligroin-alcohol mixture 5:1 (Levene) and alcohol added till no further precipitation occurs. The supernatant fluid is evaporated *in vacuo* and precipitated with acetone. The cadmium compounds were obtained in nicely crystalline form by dissolving 20 gm of the crude product in 100 cc benzene and adding a solution of 150 cc cadmium chloride in alcohol-water (7 gm cadmium chloride were dissolved in 3 cc water and 150 cc alcohol). The precipitate, which immediately settled down, was centrifuged after standing for $\frac{1}{2}$ hour and washed once with benzene—alcohol (1:1) and twice with ether (the cadmium compound is slightly soluble in these solvents). Then the cadmium compound was suspended in warm chloroform and decomposed with 25% methylalcoholic ammonia. The product thus obtained is nearly colourless. The purification was repeated three times in the same manner. All products were hygroscopic.

RESULTS OF TITRATIONS

The titrations were carried out in 25 cc of the solvent described above with N/50 Na-ethylate or N/50 $HClO_4$ in the same solvent. Five drops of indicator solution were added. With phenolphthalein the end point was taken as the first appearance of a red-violet colour and with dimethyl yellow as the formation of a distinct red colour. Blank titrations were always carried out to determine the acid- and base-binding values of the solvent used.

Lecithin

Alkaline side	N/50 Na-ethylate	Phenolphthalein		
	Titrated	Control	Difference	
0·0207 gm	0·24	0·26	—	
0·0585 gm	0·24	0·25	—	

No base binding power could be observed in lecithin. The same result was obtained using the indicators thymol blue and trinitrobenzene.

Acid side	N/50 HClO$_4$	Dimethyl yellow		Equivalent weight
	Titrated	Control	Difference	
0·0533 gm	3·16	0·17	2·99	890
0·0520 gm	3·13	0·17	2·96	880
0·0445 gm	2·82	0·16	2·06	840
0·0378 gm	2·34	0·16	2·18	865

The theoretical molecular weight of the lecithin which changes with the fatty acids of its molecule is about 800. Thus, the lecithin binds one equivalent of H+-ions when titrated with acids.

Cephalin

Alkaline side	N/50 Na-ethylate	Phenolphthalein		Equivalent weight
	Titrated	Control	Difference	
0·0498 gm	3·28	0·20	3·08	810
0·0468 gm	3·12	0·28	2·84	825
0·0643 gm	4·45	0·36	4·09	785

Acid side	N/50 HCl O$_4$	Dimethyl yellow		Equivalent weight
	Titrated	Control	Difference	
0·0568 gm	3·25	0·17	3·08	920
0·0465 gm	2·82	0·16	2·66	875
0·0435 gm	2·53	0·16	2·37	920

The theoretical molecular weight is about 770. The titration results show that about one equivalent of H+-ions is given up in titration with alkali. The equivalent weight found in titrations with acid, however, is remarkably higher than the theoretical value. This is explained by the fact that, at present, cephalin preparations cannot be made of the same degree of purity as lecithin. The molecular weight of our preparation, calculated from the P-content is 862; this value is in better agreement with the results of the acid titration and it can be concluded that one equivalent of H+-ions is absorbed at acid reaction.

Lysolecithin

Alkaline side	N/50 Na-ethylate	Phenolphthalein	Interpreted as NH$_2$—N	
Titrated	Control	Difference	mg	%
0·0485 gm .... 1·27	0·26	1·01	0·283	0·58
0·0313 gm .... 0·93	0·20	0·73	0·205	0·65
0·0320 gm .... 0·92	0·20	0·72	0·201	0·63

On the alkaline side only a small amount of base is absorbed, which corresponds exactly to the NH_2—N content found by the van Slyke method (0·60%). Obviously in the lysolecithin-lysocephalin mixture only the lysocephalin is titrated, whereas the lysolecithin cannot be titrated.

Acid side	N/50 HCl O_4		Dimethyl yellow	Equivalent
	Titrated	Control	Difference	weight
0·0266 gm	2·47	0·16	2·31	575
0·0296 gm	2·61	0·16	2·45	605
0·0250 gm	2·30	0·20	2·10	595

The theoretical molecular weight is about 520. One equivalent of H^+-ions is taken up on the acid side.

Sphingomyelin

Sphingomyelin I was made by the method of Levene and sphingomyelin II further purified by the cadmium method.

Alkaline side	N/50 Na-ethylate		Phenolphthalein	Apparent % of the total amount
	Titrated	Control	Difference	
0·0437 gm sph I	0·67	0·21	0·46	16·7
0·0470 gm „	0·73	0·26	0·47	16·0
0·0489 gm „	0·76	0·26	0·50	16·4
0·0375 gm sph II	0·35	0·20	0·15	6·4
0·0435 gm „	0·37	0·20	0·17	6·25

Acid side	N/50 HCl O_4		Dimethyl yellow	Equivalent
	Titrated	Control	Difference	weight
0·0395 gm sph I	2·13	0·16	1·97	1000
0·0406 gm „	2·19	0·16	2·03	1000
0·0371 gm sph II	2·35	0·16	2·19	850
0·0415 gm „	2·71	0·16	2·55	815

The higher the degree of purification the more nearly does sphingomyelin approximate to lecithin in its titration values. In acid reaction one equivalent of H^+-ions is bound, and the equivalent weight found by titration becomes equal to the theoretical value (about 800). At the same time the group, which can be titrated with alkali disappears and only a small residue is left. Though further purification does not remove this residue, it is assumed that it is due not to the sphingomyelin, but to

traces of acid impurities (compare the results of determination of the isoelectric point in Chain and Kemp (1934)).

DISCUSSION

A survey of these experiments shows that all the phosphatides have the common property of binding 1 mol of H^+-ions in acid reaction. In alkaline reaction they give up H^+-ions only so far as they possess NH_2-groups. This behaviour suggests that one should correlate the acid titration to the free phosphoric group and the alkaline titration to the NH_2-group. From this it is concluded that the phospholipins are essentially " zwitterions." Two types must be distinguished:

$$RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.N(CH_3)_3^+}{\overset{-O^-}{}} \qquad RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.NH_3^+}{\overset{-O^-}{}}$$

<div align="center">

I   II

Lecithin, Lysolecithin, Sphingomyelin   Cephalin, Lysocephalin

</div>
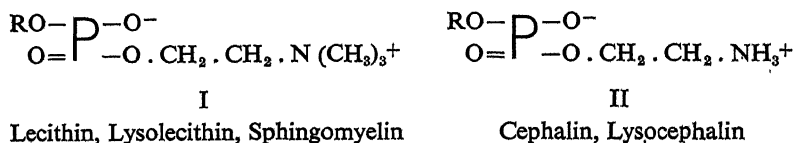
The type I (choline type) contains one molecule water less than in the formula given for the electrically neutral molecule (*cf.* p. 239), while in the molecule of type II (colamine type) such a difference does not exist.

Both types show the same behaviour with acids:

$$RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.N(CH_3)_3^+}{\overset{-O^-}{}} + H^+ \rightarrow RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.N(CH_3)_3^+}{\overset{-OH}{}}$$

$$RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.NH_3^+}{\overset{-O^-}{}} + H^+ \rightarrow RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.NH_3^+}{\overset{-OH}{}}$$
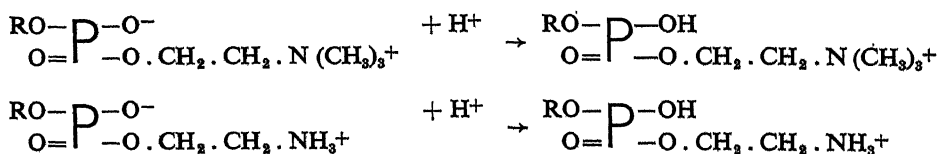
With bases, on the other hand, only the primary amines react:

$$RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.NH_3^+}{\overset{-O^-}{}} + C_2H_5O^- \rightarrow RO-\underset{O=}{\overset{}{P}}\underset{-O.CH_2.CH_2.NH_2}{\overset{-O^-}{}} + C_2H_5OH$$
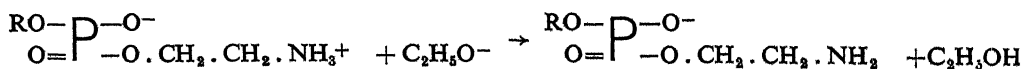
whereas a corresponding reaction obviously cannot occur with the quaternary ammonium base. The quaternary ammonium cation $N(CH_3)_3^+$ has, unlike the primary, secondary, and tertiary amines, no acid character and cannot give up H^+-ions. This conception is, in the main, identical with the older statement that quaternary ammonium bases are very strong like the alkaline hydroxide bases. The titration of the phosphoric acid group becomes possible as a result of the decrease of its acidity strength in benzene; in alcohol or water the difference in

strength between the first phosphoric group and the titration acid is too small to give a reliable indicator change. On the other hand, the titration of the amino group against phenolphthalein becomes possible because the range of colour change of this indicator (which is a weak acid) is shifted in benzene further towards the alkaline side, whereas in water the titration of ammonium salts cannot be made against phenolphthalein. The fact that weak acids being still weaker in this solvent are better titrated in benzene against phenolphthalein is also based upon this shift of the colour change range of this indicator.

Bjerrum (1923) has shown that the ratio of zwitterions and neutral molecules can be calculated from the dissociation constants of the ampholytes. In a similar way an expression can be derived using the terminology of Brönsted. Let an ampholyte have the two dissociation constants K_1 and K_2; their values, according to Brönsted, are referred to H^+-ion concentrations. Furthermore, it is assumed that each group has a dissociation constant which is independent of the dissociation state of the other group. This assumption is justified if the two groups are not too close to each other. Let the ampholyte be called A; it can occur in the forms A, $^+A^-$, ^+A, A^-, *i.e.*, as neutral molecule, zwitterion, cation, or anion. The " true " dissociation constant of the acid group is

$$K_a = \frac{^+A^- \cdot H^+}{^+A} = \frac{A^- \cdot H^+}{A}, \tag{1}$$

the dissociation constant of the basic group

$$K_b = \frac{A^- \cdot H^+}{^+A^-} = \frac{A \cdot H^+}{^+A}. \tag{2}$$

The constants experimentally found, which may be called " apparent " constants, K_1 and K_2, are complex terms to which both A and $^+A^-$ contribute.

$$K_1 = \frac{(A + {}^+A^-) \cdot H^+}{^+A} \qquad K_2 = \frac{A^- \cdot H^+}{A + {}^+A^-} \tag{3}$$

$$K_1 > K_2$$

$$K_1 = K_a + K_b \qquad K_2 = \frac{K_a \cdot K_b}{K_a + K_b}. \tag{4}$$

The ratio of zwitterions to neutral molecules follows from (1) and (2)

$$\frac{^+A^-}{A} = \frac{K_a}{K_b}.$$

If $K_a > K_b$, the zwitterions dominate, if $K_a < K_b$ the neutral molecules dominate.

If the acid constant K_a is much larger than the constant of the basic group K_b, according to (4) one gets:

$$K_1 = K_a \quad \text{and} \quad K_2 = K_b,$$

and zwitterions almost alone exist. If K_b is much larger than K_a, one gets

$$K_1 = K_b \quad \text{and} \quad K_2 = K_a$$

and the neutral molecules dominate.

If $K_a = K_b$, the ratio of zwitterions to neutral molecules is 1, furthermore from (4)

$$K_1 = 2K_a = 2K_b \qquad K_2 = \tfrac{1}{2}K_a = \tfrac{1}{2}K_b,$$

i.e., $K_1 = 4K_2$. From this it appears that K_2 can be at most $\tfrac{1}{4}K_1$. If $K_a \neq K_b$, then the difference between K_a and K_b is larger still.

Reversing this process it is possible to calculate the " true " constants from the experimental constants K_1 and K_2.

From (4) one obtains:

$$K_1 K_2 = K_a \cdot K_b$$
$$K_1 = K_a + K_b$$
$$K_1^2 = K_a^2 + 2K_a K_b + K_b^2$$
$$K_1^2 - 4K_1 K_2 = K_a^2 - 2K_a K_b + K_b^2 = (K_a - K_b)^2.$$

Therefore

$$K_a - K_b = \sqrt{K_1^2 - 4K_1 K_2}$$
$$K_a + K_b = K_1$$
$$K_a = \frac{K_1 + \sqrt{K_2^2 - 4K_1 K_2}}{2}$$
$$K_b = \frac{K_1 - \sqrt{K_1^2 - 4K_1 \cdot K_2}}{2}.$$

To apply these equations for the determination of the ratio zwitterions: neutral molecules in the phospholipins one has to introduce the values of the corresponding groups.

K_a, the acid dissociation constant, corresponds to the first dissociation constant of the phosphoric group (2.12). As in phosphoric acid esters the acidity strength generally increases (Meyerhof and Suranyi, 1926), the pK_1 can be assumed as about 1 (Levene, Rolf, and Simms, 1924, found in lysolecithin a pK_1 of 0·75). K_2 for the phosphatides of the colamine type can be assumed as about 10^{-10} (according to the primary amino group), while in the phosphatides of the choline type it can be

expected at about 0, owing to the very strong base nature of the quaternary ammonium base.

Therefore for the phosphatides of the colamine type one has

$$\frac{+A^-}{A} = \frac{10^{-1}}{10^{-10}} = 10^{+9},$$

for the phosphatides of choline type:

$$\frac{+A^-}{A} = \frac{10^{-1}}{ca.\ 0} = \infty \quad \phi,$$

i.e., the zwitterions are largely dominant.

These values are valid only for water. In other media the dissociation constants and consequently the ratio $+A^-$: A are different. For the lecithin molecule, however, one can assume that in all solvents only the zwitterionic (or cationic) form occurs, because in no solvent can the quaternary ammonium base possess acid properties.

On the basis of the experimental results it is possible to discuss some hypotheses which have been put forward for the structure of the phospholipins. In earlier papers these compounds, especially lecithin, have been regarded as " betaine like rings " (Willstätter and Stoll, 1918). " endosalts " (Grün and Limpächer, 1926, 1927) or anhydrides. It is now not clear how far the authors supposed a more or less stable ring structure or a more salt-like structure of the molecule. According to modern views there is no difference at all between betaine structure and endosalt form. From the works of Bredig (1894), Pfeiffer (1922), Bjerrum (1923), and Linderstrøm-Lang (1933) it follows that the betaine molecule is not a real ring, but a zwitterion or an internal salt. Terms like ring form, anhydride, betaine, endosalt are therefore only synonyms for zwitterion. The titration results described above confirm such a conception.

Thierfelder and Klenk (1930) and Klenk (1933) accept the anhydric endosalt formula for the synthetic lecithin (Grün and Limpächer 1926, 1927). For natural lecithin they prefer the " hydrate formula," because of the analytical data of the preparation. This opinion is not very convincing. The difference between endosalt form and hydrate form would involve a difference in the dissociation constants. The existence of an open hydrate form would mean that, in the molecule, both the quaternary ammonium base and the phosphoric acid group are very weak. This would be contrary to all experience. Besides, such a molecule should be capable of binding both acids and bases. The titrations described

above show only H^+-binding power. In the light of these observations the data of elementary analysis have less significance, especially if one considers the hygroscopic character of lecithin.

Contardi and Ercoli (1933) assume in a work on " enzymatic splitting of lecithin and lysolecithin " that, in a slow process which takes several days, the betaine form is converted into the open hydrate form. They base this hypothesis upon the observation that a pseudosolution of lysolecithin in water becomes more and more acid for six days. After this time the acidity remains constant. This observation is interpreted as the formation of the hydrate form, which reacts as a weak acid, from the neutral betaine form by a kind of hydrolysis.

It seems as if these authors are thinking of a relatively stable ring in the lysolecithin molecule which is opened gradually. Apart from the fact that a real ring linkage in the molecule is incompatible with the above-mentioned work on the betaine molecule, an opening of such a ring would cause a shift not towards the acid, but towards the alkaline side, since a very strong basic and a less strong acid group would appear.

Magistris (1931) in a review on lecithin does not decide the question: " How far the basic and acid hydroxyl group join to form an internal salt or anhydride in a betaine like manner is still doubtful." In another work (1929) he discusses the possibility that in lysolecithin the free basic group is linked with the free alcoholic OH-group of the glycerol:

$$\begin{array}{l}
\text{CH}_2\text{OOR} \\
| \\
\text{CHO}\text{---------------------O} \\
| \qquad\qquad\qquad\qquad | \\
\text{CH}_2\,.\,\text{O---P---O}\,.\,\text{C}_2\text{H}_4\,.\,\text{N (CH}_3)_3 \\
\qquad\quad\text{O===P---OH}
\end{array}$$
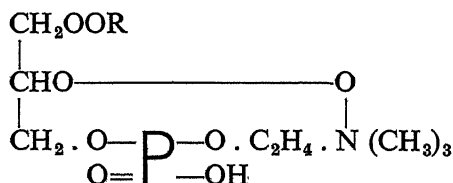
The author thinks that such a formulation might explain the greater stability of the choline in the lysolecithin molecule compared with that in lecithin. This assumption is invalid, since lysolecithin would acquire strongly acid properties and should become titratable with bases. The experiments show that no such properties exist.

Levene and Rolf (1921) discuss an arrangement of the lecithin molecule in which the remaining free hydroxyl of the phosphoric acid is condensed in an ester linking with a second diglyceride; or two monolecithins are combined into a dilecithin in the manner of a pyrophosphate. Both possibilities are rejected by the authors on account of analysis of elementary decomposition, of molecular weight of hydrolecithin (Levene and Simms,

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* In this formula there is one O-atom too many, probably a misprint.

1921) and the absence of more strongly basic properties. This conception is in accordance with the results of the titrations described above.

Another, chain-like linkage for sphingomyelin was proposed by Fränkel, Bielschowsky, and Thannhauser (1933). These authors found that in a sphingomyelin preparation which was isolated from mammalian liver, three fatty acids, palmitic, lignoceric, and stearic acids occurred always in equimolecular proportions; therefore they assume the following structural formula:

$$O=\underset{\begin{array}{c}\\ \end{array}}{P}\begin{array}{l}-OH\\ -\text{Palmitylsphingosine}\\ \diagdown OC_2H_4 \cdot N(CH_3)_3\end{array}$$

$$O=\underset{\begin{array}{c}\\ \end{array}}{P}\begin{array}{l}-O\text{————}\\ -\text{Stearylsphingosine}\\ \diagdown OC_2H_4N(CH_3)_3\end{array}$$

$$O=\underset{\begin{array}{c}\\ \end{array}}{P}\begin{array}{l}-O\text{————}\\ -\text{Lignocerylsphingosine}\\ \diagdown OC_2H_4N \cdot (CH_3)_3\end{array}$$

OH
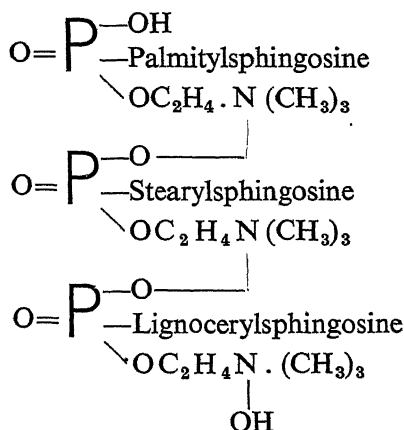
This assumption can be excluded on account of the titrations. Such a substance would bind and give up only a third equivalent of H$^+$-ions and have an equivalent weight of about 2400, while, in fact, this value corresponds to the theoretical weight of 800–850.

The titration method provides not only a suitable basis for the examination of the structure of the phosphatides, but a method of estimating the composition and purity of phosphatide preparations. This is shown especially clearly in the case of lysolecithin and sphingomyelin.

We wish to express our profound gratitude to Professor J. B. S. Haldane for his generous help in starting this work; and to Professor J. C. Drummond and Professor Sir Frederick G. Hopkins whose kind hospitality and interest made its completion possible.

SUMMARY

A titration technique is described which allows the determination of the acid and base-binding properties of the phospholipins.

The phospholipins lecithin, lysolecithin, cephalin, sphingomyelin can bind in acid reaction one equivalent of H$^+$-ions. At alkaline

reaction the phospholipins which possess a primary amino group, give up one equivalent of H^+-ions. The quaternary bases are not capable of this reaction. It is concluded that the phospholipins containing a quaternary ammonium base can exist only as cations or zwitterions, while the colamine phospholipins can exist as zwitterions, neutral molecules, anions, and cations. The ratio of zwitterions: neutral molecules is determined by the two acidity constants.

On the basis of the experimental results some hypotheses are discussed which have been put forward for the structure of the phospholipoids.

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A Study of the Correlation between the Feeding Habits and the Structure of the Hind Brain in the South Indian Cyprinoid Fishes

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Introduction

That the hind brain of fishes shows a high degree of structural variation in different forms has long been known to zoologists. Owen (1866) has stated " The primary division of the brain which consists of the medulla oblongata with the cerebellum and other less constant appendages in fishes is called " epencephalon "; it is relatively larger, occupies a greater portion of the cranium, and is more complex and diversified in this than any of the higher classes of Vertebrata." The important lobes of the medulla oblongata are the vagal and the facial lobes, which are the terminal centres for the nerve fibres of the vagal and the facial nerves respectively. The size of these lobes depends upon the extent to which their nerve fibres supply the taste buds. Herrick (1928) has pointed out that the taste buds in the pharynx are supplied by the IX and X nerves and those on the snout and the outer surface of the skin are supplied by the VII nerve. The fact that the variation in the structure of the medulla oblongata depends on the distribution of the taste buds had led Evans (1931–32) to a very instructive work on the comparative study of the brains in the British Cyprinoids in relation to their feeding habits. It is endeavoured to point out in this paper certain structural variations in the hind brain of some of the South Indian Cyprinoids in relation to their feeding habits (Cyprinidæ includes Cobitidæ also).

Material

The fishes have been collected from all the big tanks in the Mysore State, especially from the Marikanive tank (water area 34 square miles) and Sulekere (40 miles in circumference).

The brains were fixed in Bouin's fluid. A few sections were stained in hæmatoxylin and picro-indigo-carmine and others in hæmatoxylin

and eosin. The sections of the brains have been taken from behind forwards. Sections of the complete head and barbels have also been made and examined to note the distribution of taste buds.

BARBELS

The presence or absence of barbels in fishes is an important character in studying their feeding habits and the structure of the medulla oblongata. In the ground feeding fishes the barbels are used for sorting out food substances. The taste buds on the barbels are innervated by the nerve fibres of the VII nerve (Herrick, 1924). Hence those fishes which use barbels for food searching have always a greater development of the facial lobe.

Lepidocephalichthys thermalis 4 pairs of short barbels.
Nemachilus beavani 3   ,,   ,,
Nemachilichthys shimogensis   3   ,,   ,,
Barbus puckelli .... A pair of very short barbels.
Rasbora daniconius .... Absent.
Chela bacaila ....   ,,
Danio malabaricus .... A pair of very minute barbels.
Nauria danrica .... A pair of short rostrals and a pair of long maxillary barbels.

FEEDING HABITS

The factor that brings about variation in the structure of the hind brain of fishes is their feeding habits, and hence it is more appropriate to consider first the latter and then proceed to describe the structure of the brain. The freshwater fishes may be divided into two broad natural groups according to their feeding habits, viz., (1) those fishes that feed largely by taste, and (2) those which feed mainly by sight.

(1) *Fishes which Feed largely by Taste*—In the fishes belonging to this group taste buds, in whatever part of the body they may be distributed, are largely responsible for picking and sorting out food from other substances in the water. They always feed more or less at the bottom of the tank in mud or on rocky surfaces. The eyes are comparatively small in these fishes. This group can further be divided into two subdivisions.

(a) *Fishes that take in mud directly and then sort out food from inorganic substances by the help of the taste buds of the palatal organ.—Lepidocephalichthys* is a good example of this subdivision. This is a common loach in Mysore. Writing about this Sundar Raj

(1916) says " *Lepidocephalichthys* inhabits the bottom mud or sand of rivers and ponds where it usually lies buried during the day, venturing out to feed at night." The mud is taken into the mouth and there the decaying organic substances together with several kinds of algæ, which form the food of the fish, is sorted out from the mud particles by the large number of taste buds present on the palatal organ. Sections of the complete head reveals a very prominent palatal organ with comb like ridges which are thickly packed with taste buds. " During feeding this (palatal organ) is enlarged and congested with blood and serves to sort out food particles from mud " (Herrick quoted by Evans, 1931). It is stated ('Rep. Rockefeller Foundation' (1924)) that this fish is a larvivorous fish, but this is not correct.

(b) *Fish that discriminate and sort out food in mud or on rocks by the taste buds present on the barbels and the snout*—These fishes grope and grub for food. *Nemachilus beavani* (stone loach) is a very good example of this group. It is always found at the bottom of the tank or river groping and grubbing between the crevices of pebbles and rocks. *Nemachilichthys* has also the same feeding habits as the *Nemachilus*. *Barbus puckelli*, though it grubs and gropes for food, is a transitional form between this group and those which feed by sight. It is not entirely a bottom feeder and possesses a pair of short barbels which are supplied with a few taste buds. Dr. Narasimha Murty, of the health department of the Government of Mysore, tells me that *Barbus puckelli* is giving him very good results in the control of the Guinea Worm disease by devouring in great numbers the Cyclops which is the secondary host of the parasite. To show that it feeds both by sight and by taste, Dr. Murty collected a number of the outer chitinous coverings of Cyclops by washing the fæcal matter of *Barbus* and when they were introduced into a trough containing a number of these fishes they suddenly moved towards the dead coverings but turned back immediately after touching them with their barblets.

It appears to us wiser to consider (a) and (b) as subdivisions of a main group. In both the subdivisions they feed by taste and the difference between the two is that in one the taste buds are in the mouth and in the other on the barbels and the snout.

(2) *Fishes which Feed largely by Sight*—They feed on crustacea insects, and other smaller fishes more or less near or at the surface

of water.   The eyes are well developed in these forms and the mouth is directed upwards.   The barbels have become useless to these fishes and thus they have disappeared.   In some, however, there are still very small vestigial barblets.   The presence of long maxillary barbels in *Nauria* will be explained elsewhere.   The sight feeding Cyprinoids are more abundantly found in India than the ground feeding ones.   *Rasbora, Nauria,*
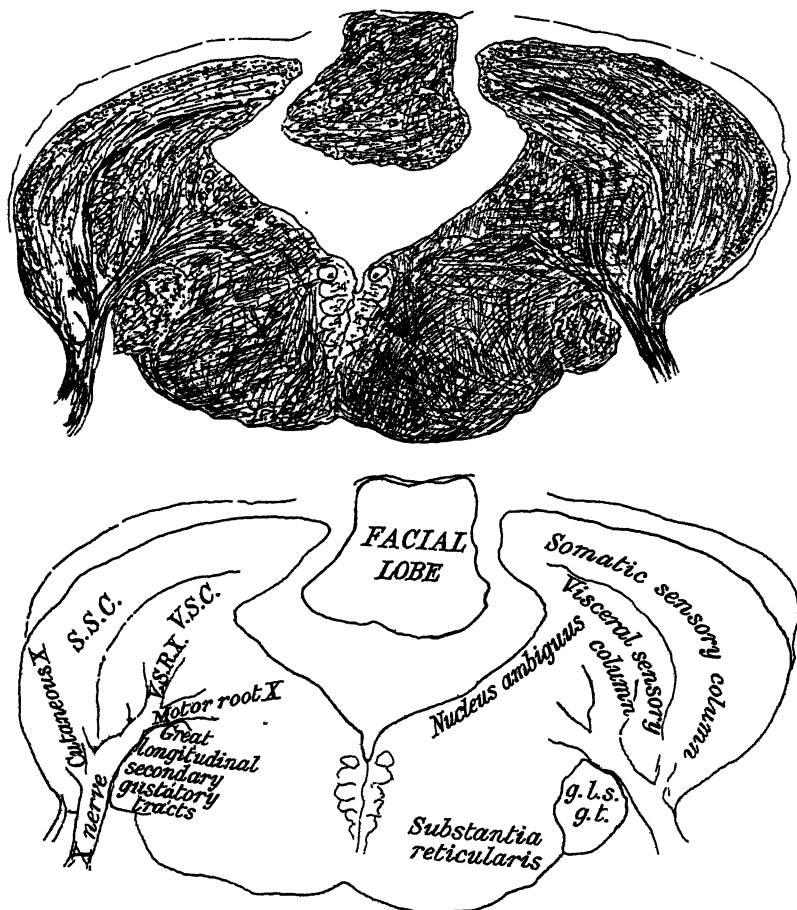
FIG. 1—*Lepidocephalichthys.*   Section across the vagal lobes

Danio and *Chela* are some of the examples belonging to this group. *Rasbora, Nauria* and *Danio* are also known to be good larvicidal fishes. The *Chela* (white carp) is a very active surface feeder.   These fish are said to feed in the cooler parts of the day—morning and evening—when they shoot into the air a number of times probably to devour the insects which may be hovering at the surface of water.   The alimentary contents

of these fishes have been examined and they contain different crustacae and insects; the latter are very conspicuous by their brownish integument.

THE STRUCTURE OF THE HIND BRAINS

A naked eye examination of the hind brains of these fishes may not show very appreciable variations but a microscopic examination of their
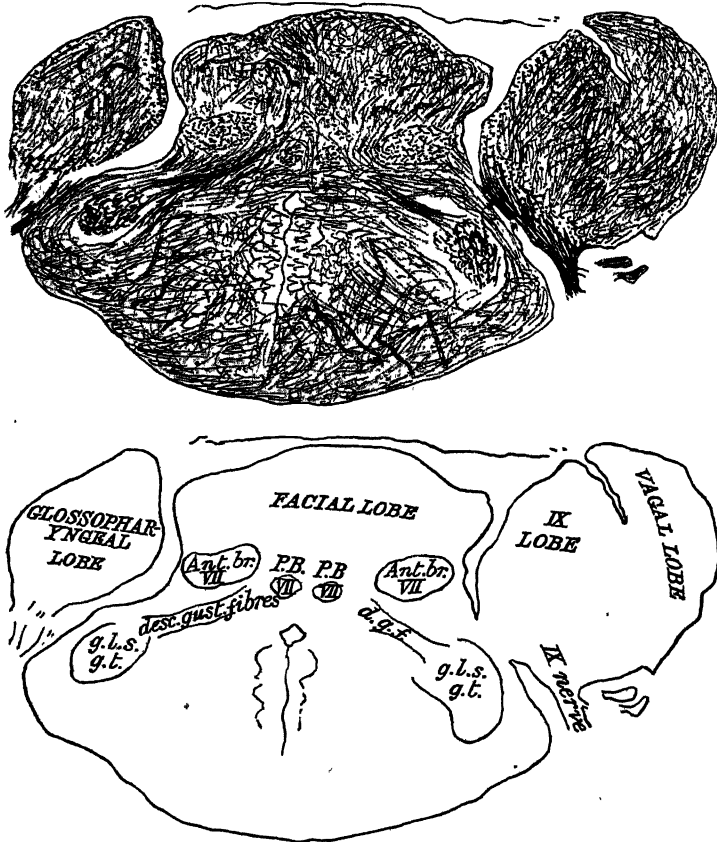
FIG. 2—*Lepidocephalichthys*. Sections through the VII and IX lobes

sections reveals remarkable differences according to their habits of feeding.

1 (a) *Lepidocephalichthys*—This fish has very prominent vagal lobes. Fig. 1 is a section of the hind brain passing through the vagal lobes at the point where the X nerve enters the brain. The brain at this point is very broad. The vagal lobes are in the form of two prominent wings embracing the posterior end of the mesially situated facial lobe. The visceral and sensory columns are well developed and the left side shows

all the branches of the vagus nerve—the superficial root, cutaneous root, visceral sensory root and the motor root—entering the vagal lobe. The section in fig. 2 shows the glosso-pharyngeal lobes and the cut ends of
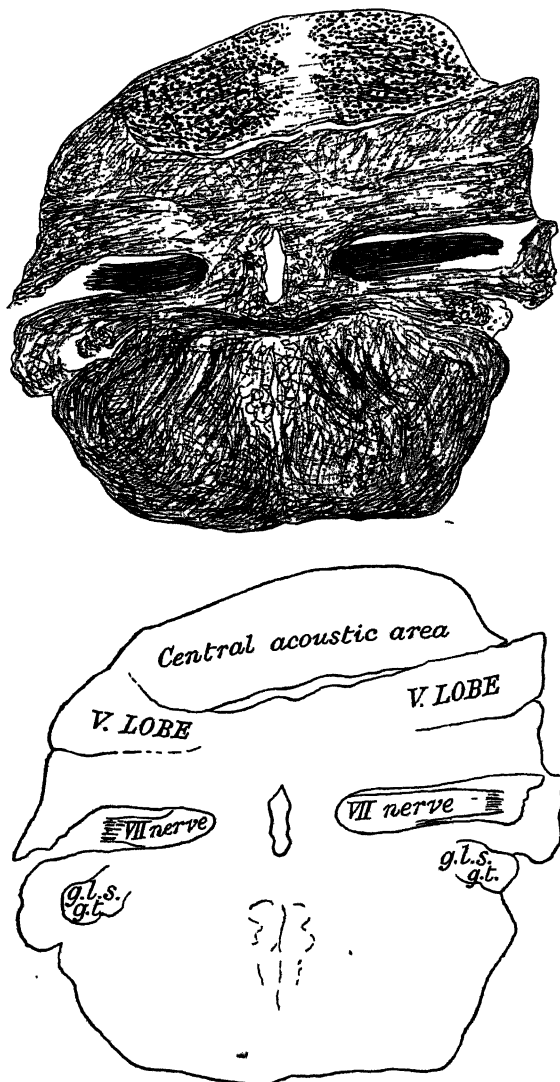
FIG. 3—*Lepidocephalichthys*

the two branches of the VII nerve. The section in fig. 3 passes through the anterior end of the facial lobes where the VII nerve are entering the brain and the round celled tissue at the base of the cerebellum—the central acoustic area.

Though this fish feeds primarily by mouth tasting, it possesses a few taste buds on the barbels as well. And the presence of these taste buds on the barbels, as in *Nemachilus*, is responsible for the division of the VII nerve into an anterior and a posterior branch.
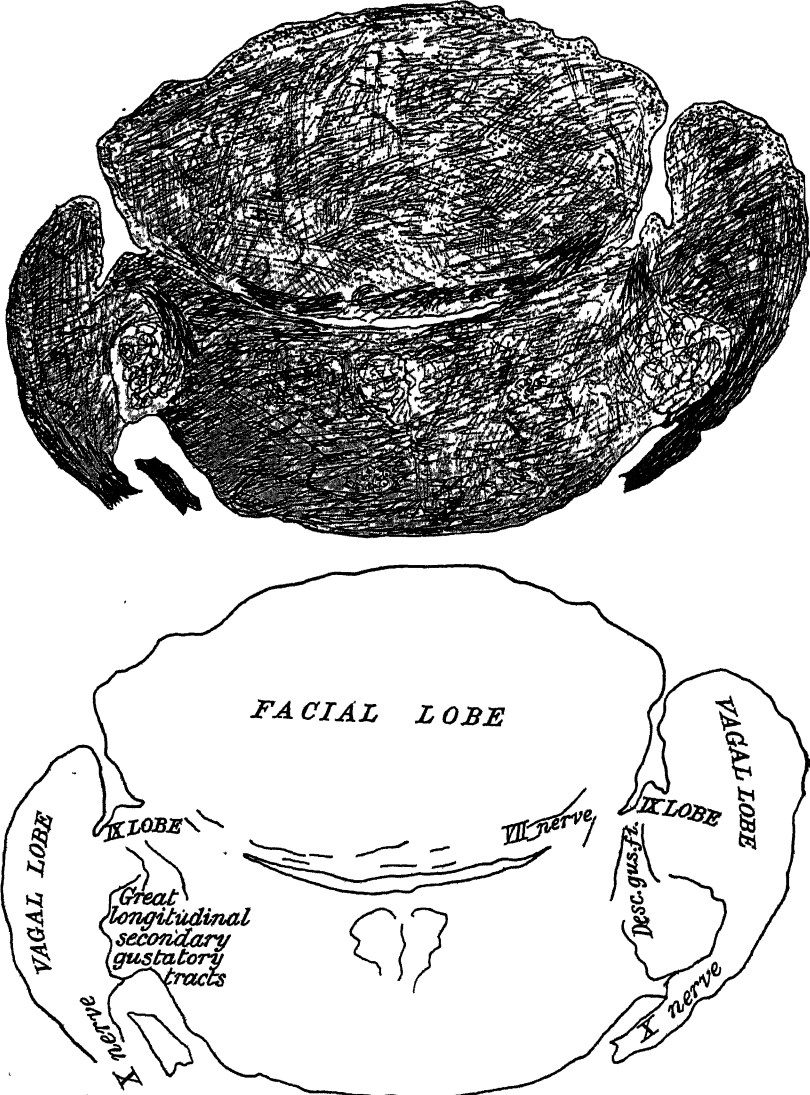
FIG. 4—*Nemachilus*

(b) *Nemachilus*—The two facials have coalesced to form a very prominent common facial lobe in the medulla oblongata of *Nemachilus*. Fig. 4 represents the section passing through the vagal lobes and the hinder part

of the facial lobe. The vagal lobes are very small as compared with those of *Lepidocephalichthys*. The facial nerve after entering the brain divides into two branches—the anterior and the posterior branches, the cut ends of which can clearly be seen in fig. 5. The anterior and the posterior
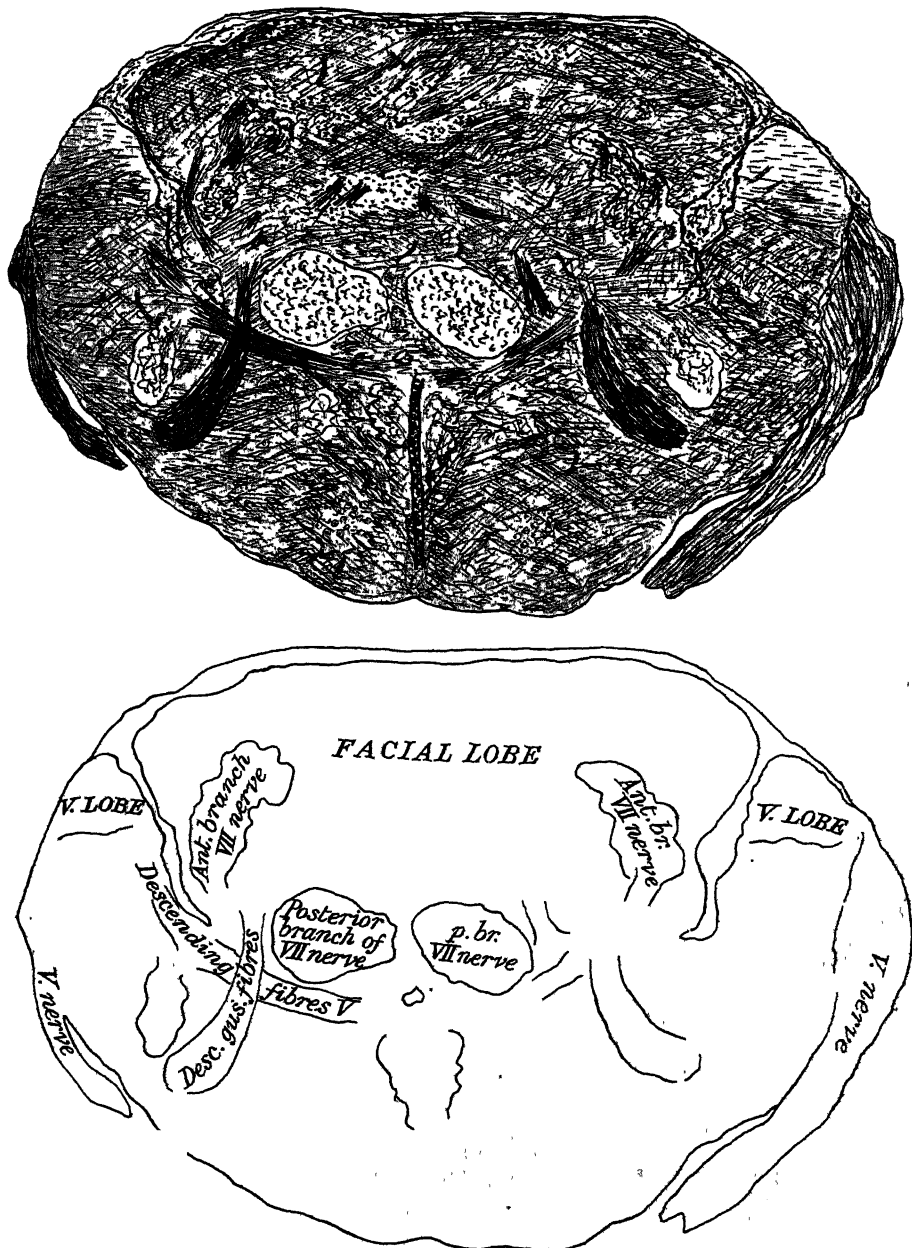
FIG. 5—*Nemachilus*

branches are separated by the descending gustatory fibres from the facial lobe. Anteriorly the facial lobes are separate as in fig. 6. The structure of the brain of *Nemachilichthys* is similar to that of *Nemachilus*.

Barbus—The vagal lobes are fairly large and the facial lobe is prominent. The VII nerve after entering the facial lobe divides into a large anterior and a minute posterior branch. The posterior branch is very small since the barbels are short and possess only a few taste buds. At the base of the cerebellum there is a well-developed central acoustic area as in the sight feeders.

2 *Rasbora* (*fig. 7*), *Nauria* (*fig. 8*), *Danio* (*fig. 9*) *and Chela* (*fig. 10*)—The brain in these fishes exhibits the structure of that of a typical surface or sight feeder. The vagals are small as also the facial lobe. The two facials have coalesced to form a common, more or less, arch-like lobe over the ventricle. The facial nerve does not divide after entering the brain. The central acoustic area which forms the base of the cerebellum in the anterior region projects backwards beyond the cerebellum in the form of a distinct central acoustic lobe. The central acoustic area as well as the central acoustic lobe are much larger in the Indian fishes than in the English forms. Groups of cells of the central acoustic area and lobe are separated by strands of nerve fibres passing outwards on either side to the octaval tubercles. From the octaval tubercles there are nerve fibres passing downwards and forming on either side an acoustic nerve.

DISCUSSION

During the course of this investigation it has been noticed that there are a greater number of surface or sight-feeding Cyprinoids in South India than ground feeders. This is probably due to two reasons: (1) that the insect fauna which forms the main food of the sight feeders is more abundant here than in the temperate countries, and (2) the insufficiency of oxygen, required for the respiration of the fish, at the bottom of the tank since the water in the tropical tanks gets readily stagnant in the hot season. Even the one mud feeder described in this paper—*Lepido-cephalichthys*—is also said to come to the surface now and then to gulp in air (Sundara Raj, 1916). Day in his introduction to Cyprinidæ (1889) remarks " Whereas Siluroids as a rule, appear to prefer muddy water, carps thrive best in those which are clear. Indian carps do not appear to restrict themselves so much to a vegetable diet as do those in Europe."

CEREBELLUM

V. LOBE | FACIAL LOBE | FACIAL LOBE | V. LOBE

VII nerve

VII nerve

V. nerve

Descending fibres of V

g.l.s.
g.t.

Great longi.sec.
gus. tracts
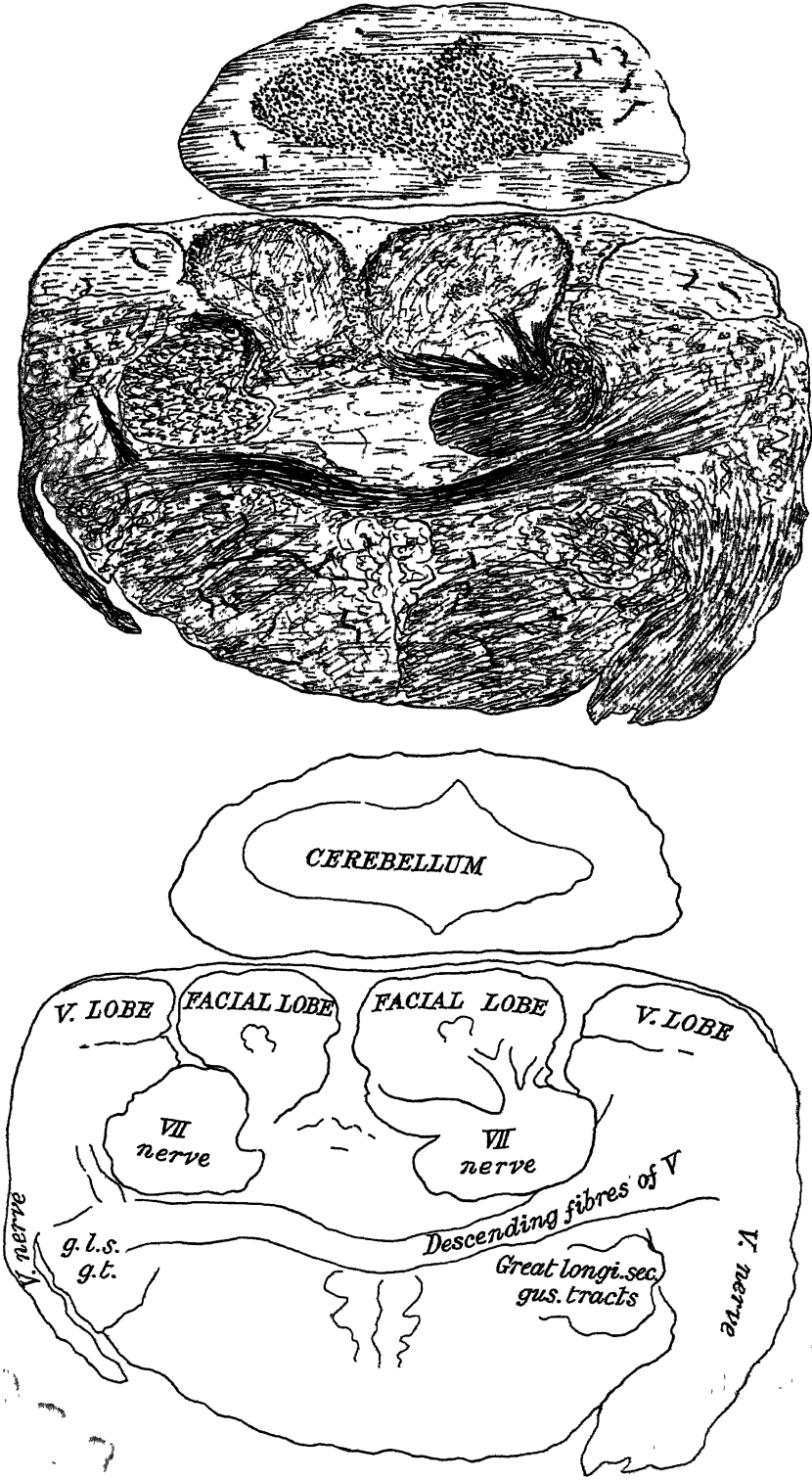
V. nerve

FIG. 6.—*Nemochilus*

The barbels in Cyprinoids and Siluroids are primarily used for searching for food particles in mud. (In all the fishes in which the barbel is gustatory in function the VII nerve divides into two or more branches after entering the facial lobe. In the surface feeders the barbels are no longer of any use to the fish and hence they are absent or highly vestigial. It was thought that the two pairs of barbels of *Nauria*—an advanced sight feeder—could not have any gustatory function and when the barbels were
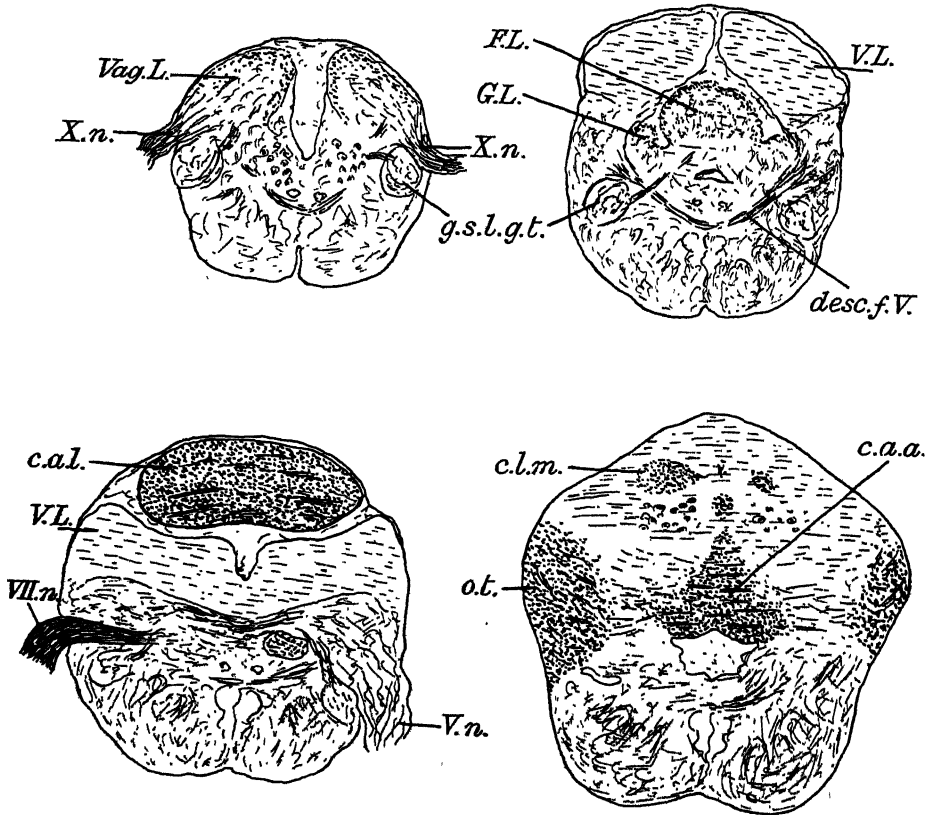
FIG. 7—*Rasbora*

sectioned and examined it was noticed that they did not possess any taste buds. Moreover, the VII nerve does not show any division in the facial lobe though the maxillary barbels of the fish are very long. Probably as Norman (1931) suggests they may receive impressions indicating approach of other fishes, or act as decoys.

It is needless for us to discuss here the function of the central acoustic area or lobe since Evans (1932) has dealt at length with its relation to the sense of hearing. The acoustic area or lobe is the terminal centre in the

brain of the auditory function. This area is very prominently developed in all the sight feeders, and fairly well developed in the ground feeding fish which comes to the surface to take in air (*Lepidocephalichthys*). But in the purely ground feeding fish (*Nemachilus*) which is not exposed to the influence of the external sound waves, this area is almost completely absent. Compared with the central acoustic area of the British plankton feeding fishes such as the roach and the bleak (Evans,
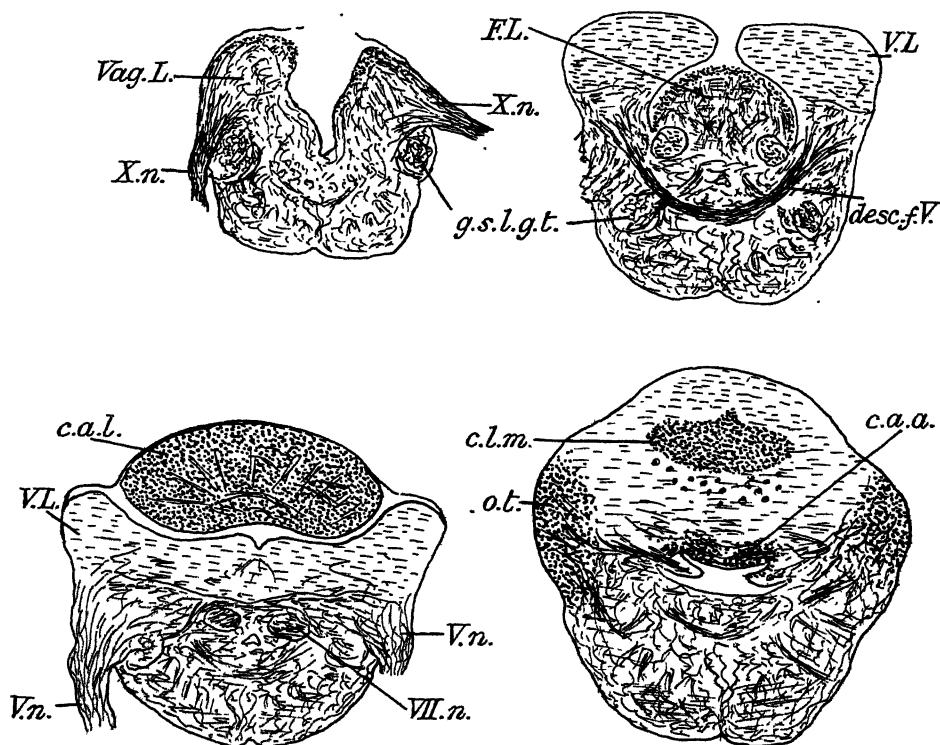
Fig. 8—*Nauria*

1932) the Indian forms like *Rasbora*, *Nauria*, *Danio*, and *Chela* have not only a larger central acoustic area but also an extension of this area behind the cerebellum in the form of a distinct central acoustic lobe. This is evidently due to a more perfect sight feeding habit of the tropical fishes, and their consequent exposure to the effects of the external sounds. In the accessory air breathing Cyprinoids and Siluroids, the extent to which the central acoustic area or lobe is developed gives a strong indication of their air breathing habit.

SUMMARY

The Cyprinoids, according to their feeding habit, can be divided into two main groups.

1. *Fishes which Feed by Taste*—These fishes feed at the bottom of the tank and possess small eyes. They can be subdivided into :—

(a) *Fishes that Feed by Mouth Taste*—Example, *Lepidocephalichthys*. This fish swallows mud directly into the mouth and there the
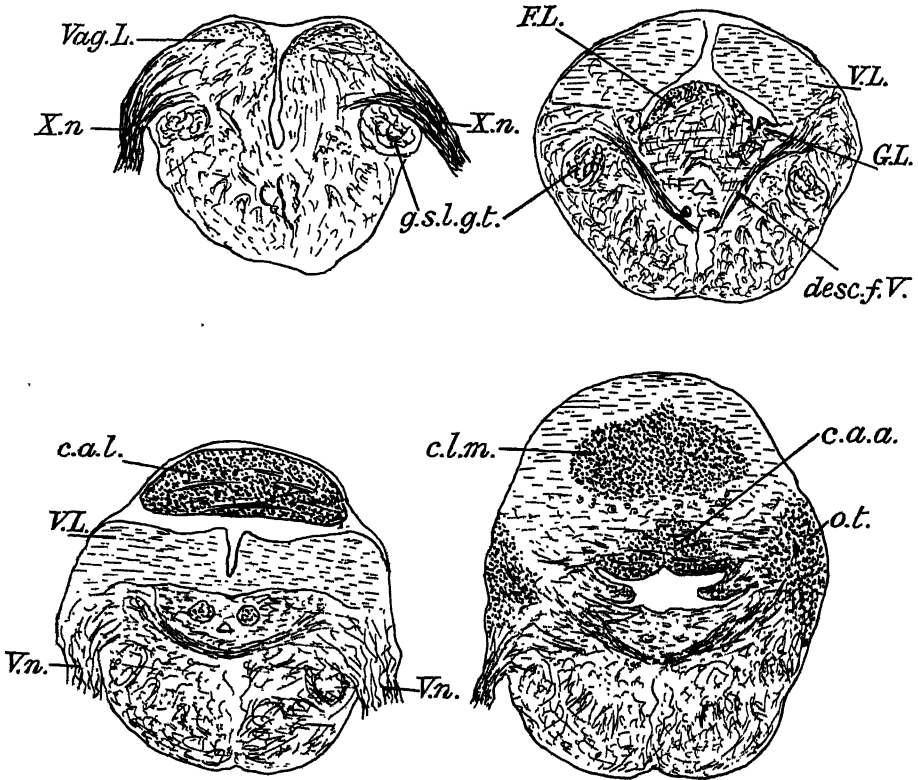
FIG. 9—*Danio*

palatal organ (closely packed taste buds in pharynx) sorts out food from other substances.   The innervation of the palatal organ is by the X nerve, and hence there is a very prominent development of the vagal lobes in this fish.   The facials are small.

(b) *Fish that Use their Barbels to Sort out Food*—Example, *Nemachilus* and *Nemachilichthys*. They grope and grub for food on the

bottom. The taste buds on the barbels are innervated by the VII nerve and consequently the facial lobe is highly developed. The facial nerve is divided into, generally, two branches after entering the brain. The vagal lobes are small.

2. *Fishes that Feed Largely by Sight*—Example, *Rasbora, Nauria, Danio,* and *Chela.* They feed largely by sight near the surface. The eyes are large and the mouth is directed upwards. They feed mainly
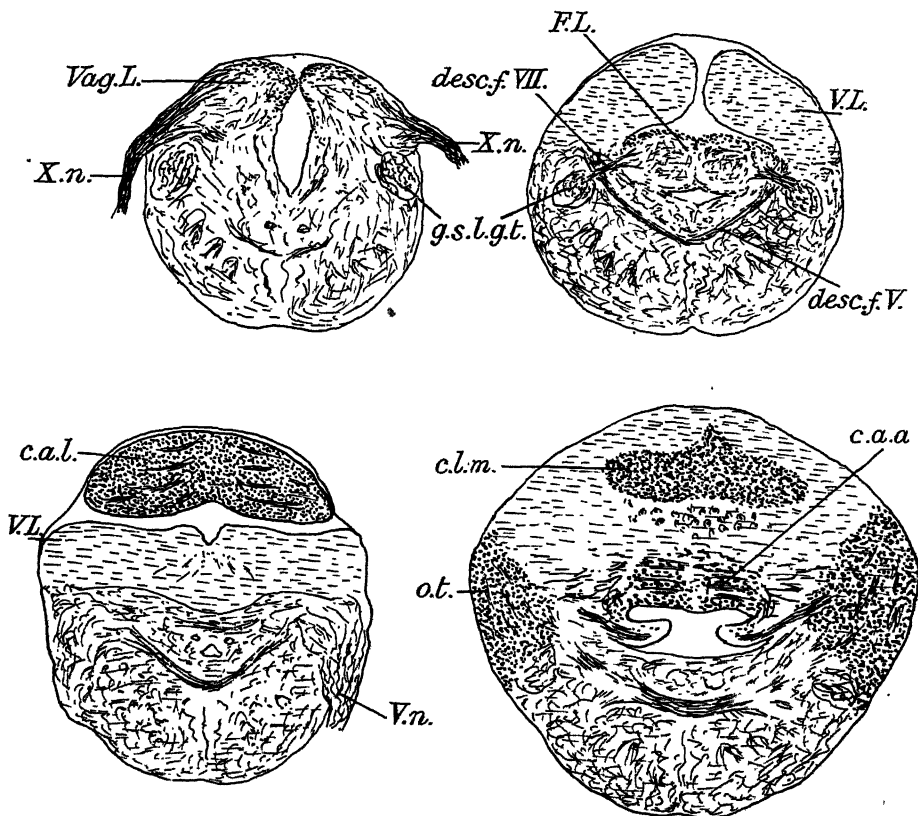
FIG. 10—*Chela*

on crustacea and insects. Barbels are absent or vestigial. They possess a well developed central acoustic lobe. Both the vagal and the facial lobes are very small.

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ABBREVIATIONS

c.a.a., central acoustic area.
c.a.l., central acoustic lobe.
desc.f.V., descending fibres of the V lobe.
desc.f.VII., descending fibres of the facial lobe.
F.L., facial lobe.
G.L., Glossopharyngeal lobe.
g.s.l.g.t., great secondary longitudinal gustatory tracts.

o.t., octaval tubercles.
Vag.L., vagal lobe.
V.L., V lobe.
V.n., V nerve.
VII.n., facial nerve.
X.n., vagus nerve.

The Kinetics of Hæmolysis in Cell-Taurocholate-Serum Systems

By Eric Ponder and Albert S. Gordon

(From the Biological Laboratory, Cold Spring Harbor, and Washington Square College, New York University)

It is well known that the serum and plasma proteins usually produce inhibition of hæmolysis by saponin, the bile salts, the soaps, and other related lysins. As early as 1908, however, Sachs described an acceleration of hæmolysis as occurring when serum or plasma is added to systems containing sodium oleate, after the oleate had been in contact with the cells for some time. The same effect was later observed, under certain conditions, by Ponder for sodium glycocholate (1922), for sodium taurocholate (1923), and for stearates and oleates (1924), and also by Sen and Mitra (1928) in systems containing taurocholate, although Sen and Sen (1928) had previously failed to obtain it. It is known from these investigations that the concentration of taurocholate, etc., used, the

quantity of serum or plasma added, and the time for which the lysin is allowed to react with the cells determine whether, and how great, an acceleration replaces the more usual inhibition, but otherwise the kinetics of hæmolysis in these systems is obscure. They are of interest, however, because of their resemblance to colloidal silicic acid-complement and brilliant green-serum systems, in which the cells react with the lysin only after being acted on by a sensitizing agent, and in which, as in these systems, the order of addition of the various components largely determines the final result (see Ponder, 1928, 1932, *a*, 1933). It has been already suggested, indeed, that the taurocholate acts by sensitizing the cells' to the lytic action of the subsequently added serum, as well as by producing lysis itself, just as brilliant green brings about a similar sensitization, and is, in sufficient concentration, a lysin *per se* (Ponder, 1934, *a*).

The only other suggestion which has been put forward is that of Sen and Roy (1930–31), who observe that either an inhibition or an acceleration can be obtained by adding various amines to systems containing sodium taurocholate, the result depending on whether the addition is made before or after the taurocholate has come into contact with the cells. Since the addition of the amines makes the systems containing cells and taurocholate more alkaline (the p_H of such systems usually being between $5 \cdot 0$ and $6 \cdot 0$), Sen and Roy suggest that the similar accelerating effect of serum may be due, in part at least, to its alkali content.

In this paper we shall describe the results obtained by the same kind of methods as have already been applied to similar systems.

1—TAUROCHOLATE CONSTANT, SERUM VARYING

In order to obtain the acceleration or inhibition which results from the addition of a quantity of serum to a system containing cells and sodium taurocholate as a function of the quantity of serum added, one proceeds in the following way.

A $0 \cdot 25$ standard red cell suspension is prepared by suspending the thrice washed cells from 1 cc of blood in 80 cc of $1 \cdot 0\%$ NaCl (saline). Most of the experiments described in this paper were carried out with rabbit red cells, but similar results can be obtained with the cells of man, the ox, or the sheep. One concentration of sodium taurocholate, *e.g.*, 1 in 20,000, is selected, and the salt may be dissolved either in $1 \cdot 0\%$ NaCl or in a suitable buffer. To $0 \cdot 8$ cc of this dilution of taurocholate is added $0 \cdot 4$ cc of the cell suspension, and the time T for complete lysis

at 30° C is determined in the usual way. Suppose that it is 5 mins. To 0·8 cc of the selected dilution of taurocholate is added 0·4 cc of the cell suspension, as before, and the two are allowed to react at 30° C for a constant fraction of the time in which there would be complete hæmolysis, e.g., if the latter time t is 5 mins, for 0·4t, or for 2 mins. At the end of this " sensitization period," which will be denoted by S, 0·8 cc of saline are added, and the time for complete hæmolysis, commencing from the moment of the addition, is determined.

The procedure is then repeated, 0·8 cc of the taurocholate being allowed to react with the cells for the same time S, at the end of which 0·8 cc of serum previously diluted 1 in 2, 1 in 5, 1 in 10, .., 1 in 100, .., 1 in 64,000, is added instead of the 0·8 cc of saline. In each case the time T for complete hæmolysis is found, always starting from the end of the sensitization period, i.e., from the moment of the addition of the serum. In this way we obtain a series of values of times for complete hæmolysis corresponding to the addition of varying quantities of serum at the end of a constant sensitization period to systems containing a constant amount of taurocholate and a constant number of cells. We also have one unique time corresponding to the addition of 0·8 cc of saline to the system, or " no serum." If the time for lysis corresponding to any particular quantity of added serum is shorter than this, there is an acceleration, if longer an inhibition.

Typically we find both acceleration and inhibition, the former occurring when the quantity of added serum is great, and the latter when the serum is highly diluted. Table I shows the results found with a taurocholate dilution of 1 in 20,000, a 0·25 standard rabbit red cell suspension, a sensitization period of 0·4t, and the addition of rabbit serum in various dilutions.

TABLE I

Serum, 1 in	2	5	20	80	200	400	600	800	No serum
T, mins....	1·0	1·8	2·5	4·0	6·5	16	42	6·0	5·0

In the presence of serum, in dilution less than 1 in 80, there is acceleration, but when the dilution is greater the acceleration is replaced by an inhibition which, however, reaches a maximum at a serum dilution of about 1 in 600. Smaller quantities of serum give less than maximum inhibition, and finally when the dilution is about 1 in 800, the addition has virtually the same effect as that of an equal volume of saline. These results appear as one of the curves in fig 1, and Sen and Mitra (1928) obtained a very similar type of curve.
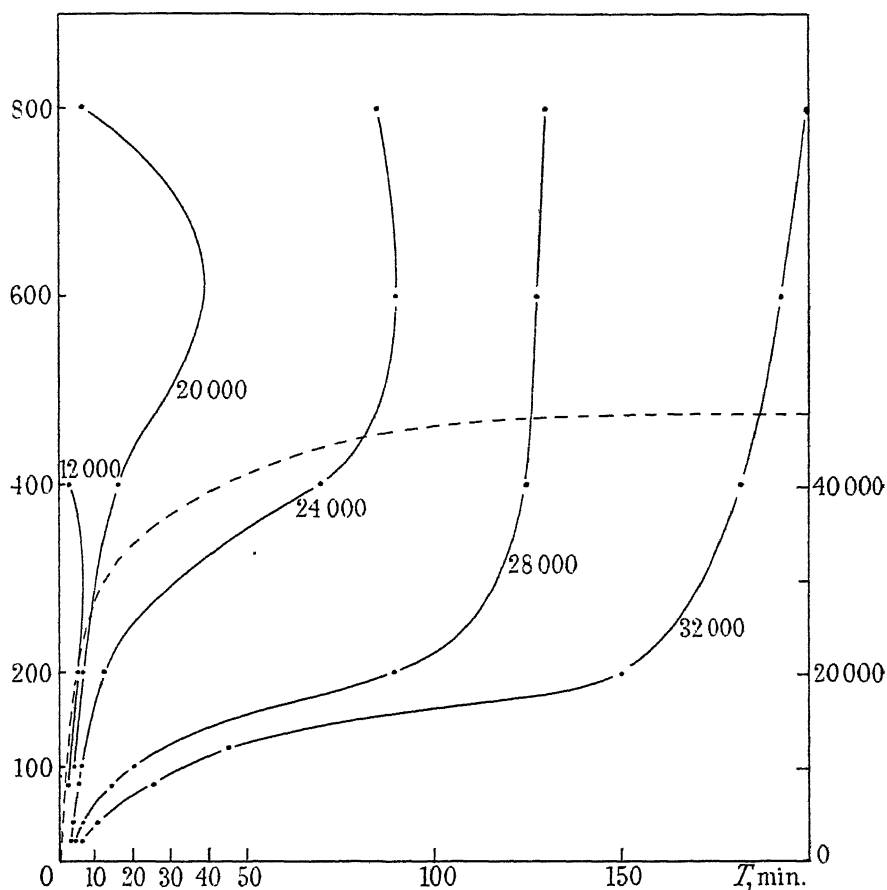
Fig. 1—Ordinate, serum dilution; abscissa, T in mins. Dilution of taurocholate is marked opposite each curve. The scale on the right applies to the dotted curve, which shows the relation of T to the dilution of taurocholate if no serum is added to the systems. This dotted curve really lies in a third dimension

TABLE II

Taurocholate, 1 in

Serum, 1 in	32,000	28,000	24,000	20,000	12,000	4,000
5	4·5	3·7	2·5	1·8	1·5	0·2
20	6·0	4·7	3·0	2·5	2·0	0·7
40	10	6·5	3·5	3·0	2·2	0·8
80	25	14	5·0	4·0	3·0	1·0
100	45	20	6·0	4·5	5·0	2·0
200	150	90	12	6·5	6·0	1·5
400	182	125	70	16	3·0	0·9
600	193	128	90	42	—	—
800	200	130	85	6·0	—	—
1600	220	118	7·5	—	—	—
3200	160	11	—	—	—	—
6400	20	—	—	—	—	—
No serum	15	10	6·5	5·0	2·5	0·8

2—TAUROCHOLATE AND SERUM BOTH VARYING

The way in which the quantity of taurocholate, the amount of serum added, and the time (in excess of the sensitization period) for complete hæmolysis are related is shown in Table II and fig. 1. These are obtained by repeating the procedure described above for a series of dilutions of taurocholate, the sensitization period being always adjusted, however, so as to be $0 \cdot 4t$, where t is the time taken to produce complete lysis of the cells by the taurocholate when neither saline nor serum is added. The entries in Table II show T in minutes.

The range of serum dilution is so great that it is necessary to plot the curves relating serum dilution to T on two scales in order to show their characteristics. The general form of the curves appears in fig. 1, in which T is shown as a function of the serum dilution for the first five dilutions of taurocholate (32,000 to 12,000) and for dilutions of serum from 1 in 5 to 1 in 800. At higher serum dilutions the curves on the right bend back towards the ordinate. The dotted curve with the subsidiary scale shows the relation of T to the dilution of taurocholate, saline only being added at the end of the sensitization period (data partly shown in the " no serum " row of Table II). In fig. 2 the points near the origin are plotted on a larger scale, and those corresponding to quantities of serum which give neither acceleration nor inhibition are shown as circles, joined by the dotted line. On any curve, points to the left of the point marked as a circle correspond to accelerations, and points to the right to inhibitions.

3—SENSITIZATION PERIOD VARYING

If the sensitization period is too short, no acceleration is observed (cf. Sen and Sen, 1928). Table III shows results for a $0 \cdot 25$ standard suspension of human red cells, taurocholate 1 in 24,000, and rabbit serum.

TABLE III

Serum, 1 in	Sensitization in 8 mins		Sensitization 3 mins	
	T	Remarks	T	Remarks
2	1·0	Acceleration	47	Inhibition
5	3·0	,,	—	—
10	5·0	,,	72	Inhibition
20	9·0	,,	107	,,
40	17	Inhibition	180	,,
80	32	,,	>300	,,
No serum	11	—	22	

From experiments of this kind it is clear that the taurocholate requires to react with the cells for some time before the conditions necessary for the acceleration are established. We take it that the establishment of these conditions is related to the progress of the " fundamental reaction " between the taurocholate and the cell component which it affects, and that only when the cell component has been sufficiently transformed as a
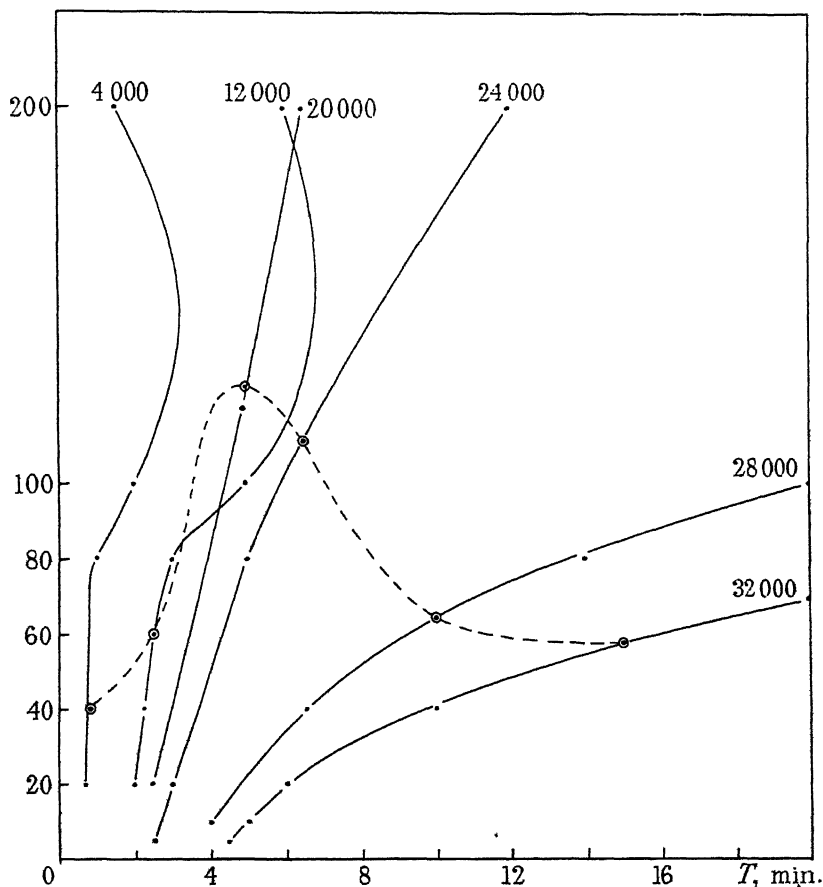
Fig. 2—The points near the origin of fig. 1, plotted on a large scale. The points shown as circles and joined by dotted line are those for neither acceleration nor inhibition

result of this reaction can the subsequent addition of serum produce lysis more rapid than that which occurs when an equivalent volume of saline is added.

4—CELL CONCENTRATION VARYING

In general, the more concentrated the red cell suspension the more difficult is it to obtain an acceleration by the addition of serum, although

it is possible to do so if the taurocholate concentration is relatively great or if the sensitization period is relatively long. Although we have not attempted to analyse the results obtained when the cell concentration is changed, we show typical results in Table IV for the sake of completeness. This table refers to systems containing rabbit red cells and taurocholate to which rabbit serum diluted 1 in 5 was added at the end of a sensitization period equal to $0\cdot3t$. The values marked (a) were obtained by adding $0\cdot8$ cc of saline at the end of the sensitization period, those marked (b) by adding $0\cdot8$ cc of the diluted serum.

TABLE IV

Taurocholate, 1 in	Sensitization period		Cell concentration		
			Standard	0·5 standard	0·25 standard
16,000	1·0	(a)	>120	170	16
		(b)	>200	>150	20
14,000	0·86	(a)	>120	62	10
		(b)	>200	>150	4·0
12,000	0·75	(a)	>120	38	7·0
		(b)	>200	124	3·0
10,000	0·65	(a)	72	20	4·0
		(b)	>100	80	2·5
8,000	0·50	(a)	46	12	3·5
		(b)	>100	72	2·3
6,000	0·40	(a)	13	5·0	3·0
		(b)	>100	15	2·0
4,000	0·25	(a)	5·5	2·0	2·0
		(b)	200	12	1·5
2,000	0·15	(a)	1·0	1·0	0·8
		(b)	0·5	0·4	0·3

In the last column (except the first two values) and in the last row there is acceleration, *i.e.*, the value corresponding to (b) is less than that corresponding to (a). This means that with this length of sensitization period and serum diluted 1 in 5 acceleration is observed only when the cell suspension is dilute or the concentration of taurocholate great. These, of course, are the conditions necessary for the " fundamental reaction " to be relatively far advanced at the end of the sensitization period. One can arrive at the same conclusion by using other dilutions of serum and other sensitization periods, always subject to the limitation that if the

sensitization period is too short or the dilution of added serum too great, acceleration is never obtained.

5—" PARTIAL " ACCELERATION

In many cases in which the result of the addition of serum at the end of the sensitization period is recorded as an inhibition, there is observed a phenomenon which has not hitherto been described, viz., a " partial " acceleration. As an illustration, take the case in which 1 in 24,000 taurocholate is allowed to react with the 0·25 standard cell suspension for 5 mins, at the end of which serum diluted 1 in 200 is added. If saline alone were added, complete lysis would result in a further 6·5 min, but when the serum is added lysis is not complete until 12 mins have elapsed
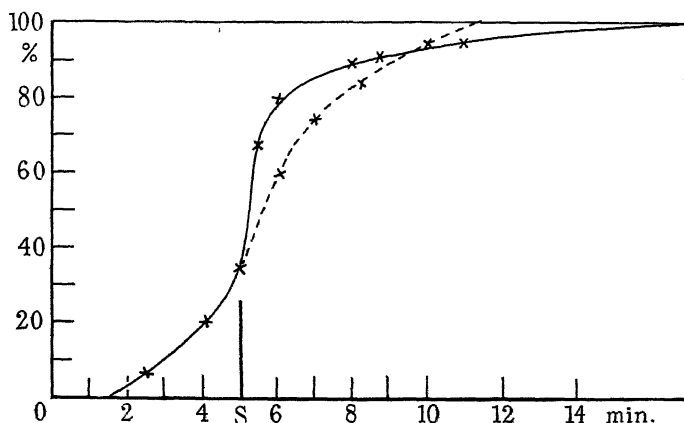
FIG. 3—Percentage hæmolysis curve showing "partial acceleration." Ordinate percentage hæmolysis; abscissa, time in minutes. Serum added at point S. The dotted curve shows the course of the reaction in the absence of added serum

(see Table II). So far as complete hæmolysis is concerned, this result is an inhibition, but what happens is actually something more complex, for within a short time (0·5 min or so) a considerable number of the intact cells in the system hæmolyse, and this is followed by a very slow lysis of the remainder. There is, in fact, accelerated lysis of a portion of the cells followed by an inhibited lysis of the remainder, and the result is recorded as an inhibition merely because complete lysis is used as the arbitrary end-point.

To reduce this phenomenon to quantitative description we clearly require to have percentage hæmolysis curves for the course of the lytic reaction which follows the addition of the serum, and these we have been unable to obtain except in isolated instances (see fig. 3). The

difficulty is that the phenomenon occurs only over a very restricted range in which the net effect is one of slight inhibition, *i.e.*, when the sensitization period is a little too short or the dilution of added serum a little too great. Speaking generally, however, it seems that the addition of a quantity of serum is capable of effecting accelerated lysis of a definite number of cells, and that the serum also inhibits the lytic action of the taurocholate which is free in the system at the moment of addition. If this number of cells is equal to or greater than the number unhæmolysed at the end of the sensitization period, complete lysis rapidly follows the addition; if, on the other hand, the number is smaller than the number of unhæmolysed cells, rapid lysis occurs until a certain definite degree of percentage hæmolysis is reached, and the remaining cells hæmolyse very slowly. To account for this qualitatively we may imagine that only a certain percentage of the cells are sensitized by the taurocholate within the sensitization period with which the phenomenon is associated, and that the added serum, acting as a subsidiary lysin (see below), can effect lysis of this sensitized number only. If the sensitization period is slightly lengthened, the " partial " acceleration is replaced by a pure acceleration, and reduction of the number of cells in the suspension, other factors being kept constant, has the same effect, both of which facts are in keeping with this explanation. Sen and Mitra (1928) observed the phenomenon, which Sen and Roy (1930–31) later associated with " ghost formation."

6—Sen and Roy's Hypothesis .

The hypothesis that the acceleration is due, even in part, to the alkali content of the added serum changing the p_H of the cell-taurocholate systems from about 6·0 to more nearly 7·0 is difficult to accept for at least two reasons: (1) essentially the same results are obtained whether NaCl at p_H 5·8–6·5, isotonic phosphate buffer at p_H 6·0, or phosphate buffer at p_H 7·0 is used to dilute the taurocholate and the serum; (2) reference to Gordon's time dilution curves for sodium taurocholate at various p_H's (1933) shows that less rapid, and never more rapid, lysis results from changing from a system at p_H 6·0 to one at any p_H greater than 6·0, but less than about 8·5. The dependence of the acceleration on the length of the sensitization period, of course, is sufficient to show that it is not the result of a change in p_H of the system alone.

The alternative hypothesis is that the taurocholate reacts with the cells, producing, if given sufficient time, complete hæmolysis, but that the result of the earlier stages of the reaction is to sensitize the cells so that the added serum acts upon them as a subsidiary lysin. Such a sensi-

tization to an ordinarily inert substance such as the serum proteins would not be unique, for it undoubtedly occurs when the sensitizing agent is brilliant green and dyes of the same class (Browning and Mackie, 1914; Mackie, 1919; Ponder, 1928, 1933), and if sensitization by taurocholate is possible when the subsidiary lysin is serum or plasma, it is not surprising that a sensitization occurs when the subsidiary lysins are substances which are lytic for unsensitized cells, such as the amines and ammonia. It is remarkable, indeed, that the list of such subsidiary lysins is as restricted as it is.

7—THEORETICAL CONSIDERATIONS

These systems differ from those which are in general like them (amboceptor-complement systems, colloidal silicic acid-complement systems, and brilliant green-serum systems) in that the substance which we consider as a sensitizing agent, taurocholate, produces measurable sensitization only when it is also actively lytic. This makes an experimental analysis of the kinetics of the systems almost impossible, for it prevents us doing what can be done in similar systems, viz., sensitizing the cells, removing the excess sensitizing agent, and then studying the effect of the subsequently added lysin (complement or serum). The situation, indeed, is very like that in which the kinetics of hæmolysis have to be studied in the presence of excess colloidal silicic acid (Ponder, 1932, *a*, sections 4 and 9), when the only thing which can be done is to make certain assumptions known to be correct for simpler systems, and then to show that on the basis of these the experimental results can be reproduced in a general way, although not numerically, for the value of essential constants is unknown and the theory requires to be too greatly simplified in order to yield results at all.

Consider a system containing 0·4 cc of cell suspension to which is added 0·8 cc of taurocholate in quantity c_0. " Initial absorption " of the lysin will occur (Ponder, 1934, *b*), and we shall have established an " external " system containing lysin in quantity c_e, and an " internal " system containing lysin in quantity c_i, where $(c_e + c_i) = c_0$. There is evidence that the changes in lysin concentration in the internal system, once it is formed, are largely independent of those in the external system (Ponder and Gordon, 1934), and we shall regard them as entirely so. During the sensitization period S a reaction between the cell component and the lysin in the internal system will proceed in such a way that after time S we shall have a quantity x_S of the cell component transformed; this will be given by

$$kS = \frac{p}{p-1}\left\{c_i^{\frac{p-1}{p}} - (c_i - x_S)^{\frac{p-1}{p}}\right\},\qquad (1)$$

and will correspond to the hæmolysis of P% of the cells of the system (see Ponder, 1934, *a*). The remainder, (100 — P)% in number, will not have undergone hæmolysis, but will have suffered an alteration of their cell component, or will have been " sensitized," to an extent exactly measured by x_S. If nothing is added to the system, or if saline only is added, the reaction will proceed until there is complete hæmolysis in time *t*, determined by substituting x_t for x_S in (1), for the mere dilution of the system with saline is supposed to affect the concentration of lysin in the external system only.*

At the end of the sensitization period S let serum in quantity Q be added to the system. The effect will be a threefold one. (1) Some of the serum will react with the free taurocholate in the external system, and some of the latter will be rendered inert. This we shall neglect as we are supposing the external and the internal systems to be independent. (2) Some of the serum will also react with some of the free taurocholate in the internal system, and will thus inhibit the reaction which would otherwise occur between the taurocholate and the cell component. This will result in an inhibition R_I. (3) Some of the serum will react with the sensitized cell component of those cells which are unhæmolysed, and this secondary lytic effect will be added to that of the taurocholate which is not inhibited as a result of effect (2). There will thus be produced an acceleration R_A, which will oppose the effect of the inhibition R_I. Because of the way in which resistance constants are measured and defined, the net effect of the simultaneously occurring acceleration and inhibition, which we can call R_N, will be (R_A . R_I), and the value of R_N can be found as follows. We refer to the standard time-dilution curve, dotted in fig. 1, which shows how T varies with c_0 when saline only is added to the systems at the end of the sensitization period, and the value of R_N is then given by the ratio

$$\frac{c_0 \text{ for time T in system with serum Q}}{c_0 \text{ for time T on standard (dotted) curve}} \qquad (2)$$

for each value of Q.

One often tries to reduce experimental results to a series of R-values in this way, but in this case the importance of doing so lies in the fact that

* There is direct evidence that this is so, for if we allow a quantity of taurocholate to react with cells for a period considerably shorter than that required for complete lysis, and suddenly dilute the system with saline, the time for complete lysis is scarcely affected. When the lysin is saponin, on the other hand (in which case there is no initial absorption), the effect of the dilution is to delay complete hæmolysis to an extent roughly corresponding to the reduction in free lysin concentration which the dilution produces.

the most likely characteristics of the acceleration and of the inhibition upon which the net effect depends are known from investigations on simpler systems.

a—*The Inhibition.* The way in which the inhibition of a lysin such as saponin or one of the bile salts depends on the quantity of serum added has been extensively studied (Ponder, 1923, 1925, 1932, *b*; Ponder and Gordon, 1934), and fig. 4 shows results for sodium taurocholate and rabbit serum plotted in terms of R-values. It is usual to plot the results another
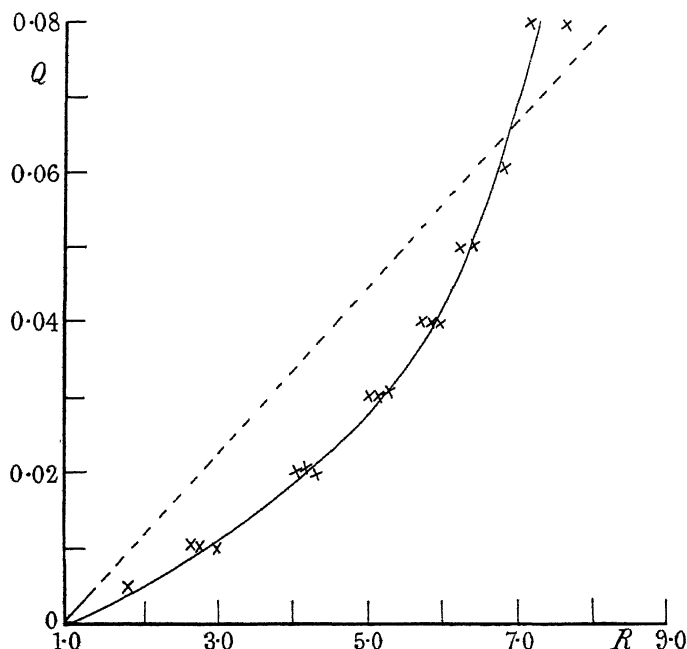
Fig. 4—Inhibition of sodium taurocholate hæmolysis by serum. Ordinate, quantity of serum Q; abscissa, R_I. Scatter of experimental points partly due to different dilutions of taurocholate being used. Dotted line is the line $1/R_A = f(Q)$, used in reconstructing fig 1

way, but a very similar relation can be obtained by a suitable plotting of the results of Ponder and Gordon (1934) for saponin. The position of the points depends to some extent on the concentration of taurocholate (or saponin) present in the system, but the relation between Q and R_I can be simplified for the present purpose by drawing a smooth curve which passes upwards from its origin at $Q = 0$, $R_I = 1 \cdot 0$ with its convexity towards the R_I-axis.

b—*The Acceleration.* In the case of the acceleration, what we have essentially is the lytic action of part of the added serum upon the sensitized

cell component augmenting the lytic action of that part of the taurocholate in the internal system which is not inhibited by the remainder of the serum, *i.e.*, the effect of one lysin (serum) added to that of another (taurocholate). If the two effects are additive, the resulting acceleration when expressed in terms of its resistance constant R can be shown to have the properties illustrated in fig. 5. The relation between Q and R_A is a curve convex to the R_A-axis, and that of $1/R_A$ to Q is a straight line which cuts the abscissa at $1/R_A = 1 \cdot 0$. The position of the curve and the slope of the line are determined entirely by the units used, *i.e.*, by the activity of the added serum as a lysin expressed in terms of the activity of the tauro-cholate as a lysin. In the present case the units are arbitrary. (For further discussion, see Ponder, 1934, *c*.)
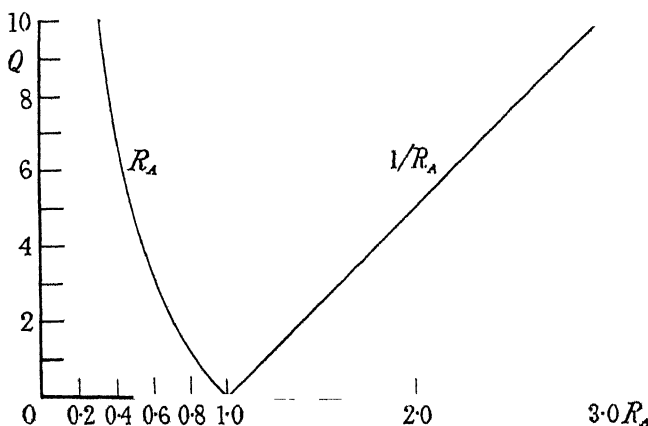
FIG. 5—R_A and $1/R_A$ as functions of Q, the latter in arbitrary units

c—The Net Effect. Since this is given by (R_A, R_I), there is inhibition for those values of Q for which $R_I > 1/R_A$, acceleration for those values of Q for which $R_I < 1/R_A$, and neither the one nor the other when $R_I = 1/R_A$. Expressed graphically, this means that when the straight line in fig. 5 lies to the left of the curve in fig. 4, there is inhibition, when the line lies to the right of the curve, there is acceleration, and for that value of Q at which the line cuts the curve, there is neither the one nor the other. As the straight line must always cut the curve at some point, we must have an acceleration when Q is great, an inhibition when Q is small, and neither when Q has some special value. This is what is found experimentally (see Tables I and II, and figs. 1 and 2).

Unfortunately no theory based on what happens in simpler systems is capable of accounting in detail for what happens when c_0 is varied. There must, for every value of c_0, be a point at which the straight line cuts the

curve, but meantime it is inexplicable that the value of Q to which this point corresponds should first decrease with a decrease in c_0, and then, after passing through a minimum, increase again (see fig. 2, in which the points are marked as circles).   When the sensitization period is varied as c_0 varies, and adjusted, as in these experiments, so as to be a constant fraction of the time required for complete lysis by taurocholate alone, the amount of transformation of the cell component at the end of the sensitization period ought to be constant and independent of c_0; the addition of a quantity Q of serum ought then to be equivalent to adding a constant quantity of a subsidiary lysin to the system, and the amount of acceleration produced thereby ought to be less as the value of c_0 is greater. Reference to fig. 2 will show that this is so for dilutions of tauro-cholate between 1 in 32,000 and 1 in 20,000, but that with greater concentrations the value of Q corresponding to neither acceleration nor inhibition increases again, *i.e.*, the slope of the line $1/R_A = f(Q)$ first increases and then decreases as the value of c_0 is increased.   It looks, in fact, as if the added serum were relatively inactive as an accelerator in the presence of very large quantities of the bile salt.   The point will be referred to again directly.

8—RECONSTRUCTION OF FIG. 1

The best test of this hypothesis is to show that it enables us to reconstruct the experimental curves shown in fig. 1, at least so far as their essential characteristics are concerned.

We shall suppose that the slope of the line $1/R_A = f(Q)$ is the same for all values of c_0, instead of first increasing and then decreasing as c_0 increases, and that this line cuts the curve $R_I = f(Q)$ (experimental data of fig. 4) at the point $R = 7 \cdot 0$.   Since the values of Q are arbitrary, the point of intersection can be put anywhere, but this point gives the best results.   The net effect of the simultaneously occurring acceleration and inhibition is computed for each value of Q, and, by means of the experimental standard time-dilution curve for taurocholate acting alone (dotted in fig. 1), is converted back into a value of T by means of expression (2).

The result is a set of curves somewhat resembling that in fig. 1, but differing from the latter in that after bending first near the origin and then back upon their course at some maximum value of T they pass upwards to approach a point corresponding to the figure in the " no serum " row of Table II only when the amount of added serum is infinitely small. The curves of fig. 1, on the other hand, approach the same values when the quantity of added serum is still appreciable (*e.g.*, 1 in 64,000, 1 in

32,000, ..., 1 in 200). The discrepancy can clearly be done away with by introducing the subsidiary assumption that when the quantity of added serum is very small it cannot enter the internal system to act as an accelerator, *i.e.*, that it is completely used up in combining with the taurocholate in the external system, which, of course, it reaches first. This means that the straight line must make a small intercept on the Q-axis.
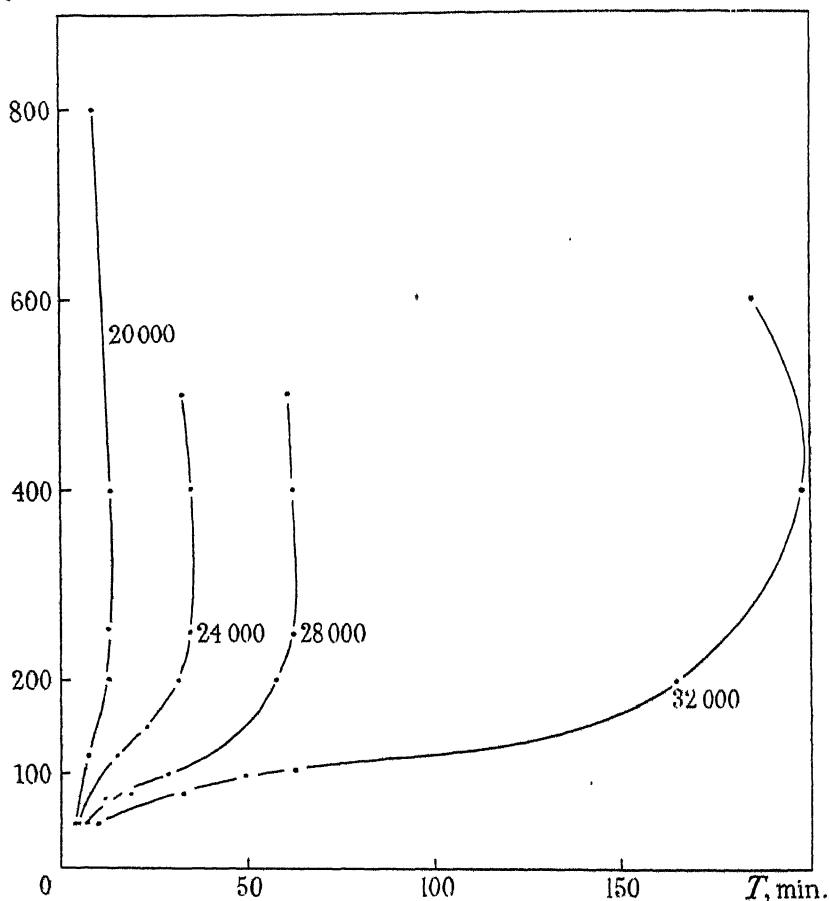
FIG. 6—Similar to fig. 1, but reconstructed from the data shown in fig. 4

Assigning to this intercept the value 0·001 in the arbitrary units used, and computing as before, we get the series of curves shown in fig. 6. These have the principal characteristics of the experimental curves, and differ from them only in the way in which the individual curves of the family are spaced when c_0 is varied. An even greater resemblance, and, indeed, quite a good fit, could be obtained by varying the slope of the

straight line and the intercept which it makes, but this would be tantamount to introducing arbitrary constants without experimental justification.

The reconstruction, of course, does not reproduce all the characteristics shown on a large scale in fig. 2, for the computations are made on the supposition that the value of Q which corresponds to neither acceleration nor inhibition remains constant. It is possible that the way in which this value behaves when c_0 is varied, *i.e.*, its decrease to a minimum and its subsequent increase is related to the same sort of phenomenon as makes it necessary to draw the line $1/R_A = f(Q)$ with an intercept on the Q-axis, for a large amount of free lysin in the external system might be relatively more effective in combining with added serum than a smaller amount might be; there is no point, however, in carrying the analysis further in the meantime.

The way in which the kinetics of these systems are affected by variations in the length of the sensitization period and in the concentration of the cell suspension are so clearly accounted for in a general way by the foregoing hypothesis that it is unnecessary to consider them in any greater detail.

SUMMARY

This paper is concerned with the kinetics of hæmolysis in the very complex sodium taurocholate-cell-serum systems, in which the addition of serum or plasma to cells which have been in contact with the taurocholate results in acceleration of hæmolysis, inhibition of hæmolysis, or neither, according to the conditions prevailing in the system at the moment of the addition. Sodium glycocholate or the soaps can replace the taurocholate, and there is no specificity connected either with the kind of cell or the kind of serum used. The way in which the final result depends on the concentration of the reactants and on the length of the " sensitization period," *i.e.*, on the time during which the taurocholate acts on the cells before the addition of the serum, has been investigated quantitatively, and it has been found possible to reproduce the essential characteristics of the experimental curves by making certain simple assumptions, viz., that the taurocholate acts on the cells partly as a lysin and partly as a sensitizing agent, the added serum then acting on the sensitized cells as a subsidiary lysin. The case seems to be very similar to that in which cells sensitized with brilliant green and other tri-phenyl-methane dyes are hæmolysed by subsequently added serum or plasma, these dyes also being, like the bile salts or soaps, hæmolysins if used in sufficient concentration. Another hypothesis, that the lytic action of the

added serum is due to its producing a change in the p_H of the taurocholate-cell systems, is rejected in favour of the foregoing on experimental grounds.

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Studies on the Nature of the Amphibian Organization Centre*

I—Chemical Properties of the Evocator

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[Plate 18]

Introduction

During the amphibian egg-laying season of 1933, Needham, Waddington, and Needham (1933, *a, b*; 1934) obtained evidence that the activity of the organization centre of the newt gastrula is partly due to the presence of an ether-soluble substance. The active ether extracts were found to be capable of evoking the formation of a neural tube from the competent presumptive epidermis of the gastrula. It seems difficult, however, to suppose that they can determine the regional character of the evoked neural plate, as normal living organizers do, and the active substance is therefore spoken of as the evocator, to emphasize the fact that its functions represent only one part of the whole process of embryonic induction.

The presence of the evocator could also be demonstrated in ether extracts of adult newt tissues; and in a research carried out at the same time Holtfreter (1933) showed that the evocator is present in a large number, if not in all, adult tissues from animals belonging to nearly all the groups of the animal kingdom. Holtfreter found that evocation occurred after the implantation of adult tissues which had been killed and treated with various solvents, but he showed that a prolonged extraction with ether tended to lessen, though it did not entirely destroy, the evocating power 'of the tissue. This result, which so far as it went was confirmatory of Needham, Waddington, and Needham's work, was, however, denied by Fischer and Wehmeier (1934), who, on repeating

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* A preliminary communication of the results reported in this paper has been made (Waddington, Needham, Nowinski, Needham and Lemberg, 1934).

the extraction experiments, could confirm the fact that the ether extracts were active, but claimed that the evocating ability of the tissues was not much lessened by the extraction. In a more recent communication (1934, *a*) Holtfreter has repeated his extractions, and finds that the activity of the extracted tissue is only slightly lowered. It is very probable, however, that there will be difficulty in extracting the whole of the active ether-soluble substances from a given mass of tissue. There is general agreement that ether extracts contain an active substance.

It cannot be taken as proved that the ether soluble evocator is the only substance which is capable of exerting these effects.

Fischer and Wehmeier (1933, 1934) have claimed that evocation can be performed by several different substances, glycogen, muscle adenylic acid, and thymonucleic acid, and this claim has been accepted in some quarters (*e.g.*, Rotmann, 1933; Edlbacher, 1934). It is clearly impossible to discuss the nature of the evocator in general terms until the question is settled. One must defer a final decision until the work of Fischer and Wehmeier is published in full, but the evidence for their view which is available up to the present is not very convincing. Among the substances named by the German authors, glycogen is the one which has been most fully investigated from this point of view. In their later paper, Fischer and Wehmeier themselves admit that it is possible, though difficult, to prepare non-active specimens of glycogen, and we shall here show that crude glycogen contains an adsorbed ether soluble substance to which it is reasonable to attribute the activity previously believed to be a property of the glycogen itself. Similar contamination may explain the activity attributed to the other substances, and it may be as difficult to remove the active material from them as it is from the dried tissue. Experiments with ether extracts of thymonucleic acid have not yet led to any definite results, since most of the experiments had to be done on *Discoglossus* eggs in which implants are rarely effective.

With these facts before us, we find it impossible to accept without question the assertion of the activity of adenylic acid and thymonucleic acid, until at least the details of the preparation and purification have been published. Similarly, the recent report of Barth (1934) of the activity of the cephalin fraction of the ether extract of brain tissue cannot be held to prove the activity of cephalin itself, as indeed Barth recognizes.

There seems, then, no good reason to suppose that there is more than one substance concerned in the different preparations of evocating substances which have been prepared by various investigators.

As will be seen in the next paper, Waddington and Needham have obtained evocations by the implantation of at least two synthetic hydro-

carbons belonging to the phenanthrene series. Since these substances were prepared by synthesis there is no possibility that they are contaminated with active substances of animal origin, although it is conceivable that they contain minute traces of other substances allied to them in chemical constitution. These active synthetic hydrocarbons are also ether soluble.

One must consider, therefore, three groups of evocating substances: (1) the active substance or substances contained in ether extracts of animal tissues, (2) the active hydrocarbons, (3) the substances mentioned by Fischer and Wehmeier, namely, glycogen, adenylic acid, and thymonucleic acid.

In this paper evidence will be presented which suggests, firstly, that the active substances mentioned in group 1 above are nearly related to those mentioned in group 2, and, secondly, that the activity of glycogen, and conceivably of the other substances mentioned in group 3, is due to contamination with substances related to those of groups 1 and 2.

MATERIAL AND METHODS

In order to test the various extracts for their evocating ability, they have to be made solid and implanted into the blastocoel of newt gastrulæ by the implantation (" Einsteck ") method of Mangold and Spemann. The ether extracts originally employed by Needham, Waddington, and Needham were solidified by being dissolved in solid triglycerides, but it was clear that the implantation of lumps of fat was not well tolerated by the embryos, and the results were not very regular. The same authors made a few experiments in which the ether extract was emulsified with a solution of egg-albumen, subsequently coagulated by heat; this drove off all traces of the solvent. A similar technique has been extensively used in the present investigations, but acetone was substituted for ether as the solvent during the emulsification, as suggested by Boyland (1932). All the important experiments have been made by this method. An earlier series was, however, made at the beginning of this laying-period, by the method of emulsifying the acetone solutions with gelatin, and then driving off the solvent by heat. With this technique, we obtained no positive evocations at all, although Fischer and Wehmeier report a high percentage of successful experiments with it. It is possible that the concentration of gelatin employed is of paramount importance, or special precautions may be necessary during the emulsification. The details of Fischer and Wehmeier's technique are not yet available.

All the coagula were prepared by one of us (D. M. N.—see next paper) under uniform conditions. The extracts were evaporated and the residue dissolved in acetone. The acetone solution was then poured into a warm solution of egg albumen, with vigorous stirring during the mixing. The mass was heated further on a water bath till all trace of acetone had gone. No attempt was made to work quantitatively, but as a rule about 1–3 mg of residue was dissolved in about $0 \cdot 5$ cc of acetone, and added to an equal volume of saturated albumen solution.

The method of emulsification in egg-albumen, although adequate, is not ideal. In the first place, control implantations of the albumen treated with pure acetone give a few cases of " slight palisades," which may be regarded as representing a first stage towards the formation of an evocated neural tube; these are more fully described later in the section dealing with the control experiments. Then, although the albumen implants, like those of gelatin and unlike those of fat, seem to exert no deleterious effects on the host embryos and are not often extruded after the implantation, difficulty is often experienced in making sure that they remain in contact with the gastrula ectoderm and do not become swallowed up and buried in the yolk during gastrulation. This difficulty is probably due mainly to the hard consistency of the implants. Finally, it may be doubted whether the albumen coagulum offers ideal conditions for the passage of the emulsified ether-soluble substances into the adjacent cells. Notwithstanding its imperfections, however, this technique seemed to be the best which we could discover.

It is probable that the conditions for diffusion are of the highest importance. Thus Holtfreter (1934, a) reports that simple cell-free extracts always induce less well than dead tissue, and this loss of inducing power must probably be attributed to physical changes in the state of aggregation of the active substance.

The experiments have been performed on four different species of Urodele, namely, *Triton tæniatus*, *Triton alpestris*, *Triton cristatus*, and the Axolotl, *Amblystoma*, and on one Anuran, *Discoglossus pictus*. These animals are indicated in the protocoll numbers by the small letters a, b, e, d and c respectively. The *Triton alpestris* were obtained from Germany and the *Discoglossus* from Algiers.

THE GRADED SERIES OF INDUCTIONS

Before proceeding to a detailed account of the experiments, it is necessary to give a summary of the sort of effects which are obtained. The Urodele reactions will be discussed first. In the most successful

experiments, the competent ectoderm of the gastrula has reacted by forming a neural tube, which, although perfectly definitely recognizable, is never entirely normal in its anatomy. The abnormalities relate chiefly to the size and position of the lumen; in the tubes which we have obtained the position of the lumen in the cross-section seems to be more or less a random one, with a tendency to be central. There is no orderly development of a thin floor and thick walls to the tube, characteristic of the trunk part of the normal neural tube, nor is the cross-section in any way characteristic of any definite part of the body. This abnormality may be due partly to the lack of the neighbouring mesodermal axial organs, which Holtfreter and others have shown to exert important effects on the shape of the tube, and partly also to the absence of the individuating effect of the living organizers. Our present data do not allow of any statement as to the relative importance of these two factors. Embryos in which a definite neural tube has been induced are placed in class A.

At the other extreme there are embryos in which no reaction at all has been provoked by the implant. These cases are most frequent among the controls, but also occur among the embryos into which active substances have been implanted.

Between these two extremes can be placed a series of types of reaction which give the appearance of having resulted from a series of quantitatively different stimuli. The lowest type in this series shows merely a thickening of the ectoderm as a whole; this type is classified as class D, in accordance with nomenclature used in Needham, Waddington, and Needham's paper (1934). The next stage is characterized by the differentiation of the inner layer of the ectoderm into a columnar epithelium with tightly packed cells containing elongated nuclei. This is the class B. The variation of its members tend in three directions: (1) the columnar epithelium may fold up into a tube and pass finally into a definite neural tube of the type mentioned above (class A); or (2) the epithelium may be many layered, and the layers may be less regular, the cells less columnar and more cuboidal and at the same time less tightly packed, and in this way a transition may be made to the type of reaction described as belonging to class E. In some specimens solid rod-like bodies of neural tissue have been found, usually embedded in a mass of tissue intermediate between classes B and E; they are assigned to class C. Finally (3) the epithelium may be many layered and break away from the surface layer of ectoderm, becoming closely adherent to the implant. The cells are elongated and in the best cases are typical neural cells, the whole mass being clearly of a neural character; even in its anatomy there is a strong tendency for the strand of tissue to roll up into a tube, but this is usually prevented by

the mechanical hindrance of the implant. Typical specimens of this type are classified in class B+++.

Of these classes, A and C are made up of specimens whose reaction can certainly be taken as positive, and when they occur they prove the presence of the evocator in the implanted material. Class B is more equivocal. It is perhaps likely that reactions of this class occur in response to the stimulus of the evocator, when the latter is available only in small quantities. But low grades of this type of reaction have been obtained among the control implantations of albumen. Probably they are due to the presence of minute traces of the evocator in our specimen of albumen, but they may perhaps represent only a general reaction to an indifferent mechanical stimulus. In any case they clearly count as negative results. On the other hand, in certain experiments very large and well-developed palisades have been obtained, so much more extensive than the control palisades that they obviously represent reactions to the implanted substances, and tending so definitely to grade into tubes or rods of neural tissue that they must be considered as positive. These various types are classified as B—, control palisades, B+, palisades better than the controls, B++, palisades much better than the control, The first sub-class counts as negative, the second as probably positive, the third as definitely positive. B+++ palisades, mentioned above, must also be taken as certainly positive.*

The three species of *Triton* are all very similar in their reactions, and can be classed together in the tables. The reactions of the Axolotl embryos probably also fall into the same classification, but owing to their greater susceptibility it has usually been impossible to keep the operated embryos alive long enough for the characteristics of the various classes of palisades to become apparent.

The reactions of the *Discoglossus* ectoderm are rather unlike those of Urodele tissue, and are not so easy to classify. Although the morphological development of the embryo proceeds very rapidly indeed, the histological differentiation of the tissues remains rather indefinite till quite a late stage, so that it is difficult and sometimes impossible to be certain of the nature of the masses of tissue which have been induced. A few experiments were made in which the blastopore region was implanted, so as to obtain some examples of the normal appearance of inductions.

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* Holtfreter (1934, *a*) has proposed a somewhat similar classification ; our types A, C and B+++ correspond to his "neural inductions," B+ and B++ to his "neuroid inductions," B — to his "faintest indications." We have not observed the induction of definite organs, which also did not occur in Holtfreter's extract experiments.

The embryos were kept alive till a late stage of development, and it was found that in some cases the superficial ectoderm of the body had become converted into neural tissue without development into a tube.   In other cases, in which the ectoderm had folded inwards to form an induced neural tube in the usual way, three or four small tubes were induced and not one large one, as is more usual in Urodeles.

These morphological irregularities, combined with the lack of histological differentiation, make it impossible to be certain of the classification of many of the results obtained.   The ectoderm lying above the implant is usually thickened to a greater or less extent.   Within this thickening large intercellular spaces may appear.   They are often lined with highly pigmented cells which in life were probably actively secreting mucus. In other cases the cells lining the spaces may show some signs of being neural in nature.   The histology, however, is not sufficiently distinctive for it to be profitable to attempt a classification of these doubtful cases. Only those specimens have been classed as positive in which the presence of neural tissue is undoubted.   The others are placed in class D or class K (see below).

Among the negative thickenings, very many show signs of differentiation into suckers, with characteristic long columnar, highly pigmented and secreting cells, accompanied by a small quantity of mesenchyme.   The histology of these suckers is the most easily recognized of any in the early stages, and is often very perfectly developed in the induced thickenings.   The ectoderm seems to be capable of reacting in this way to quite unspecific stimuli, since suckers have occasionally been induced by control implantations of albumen, and also by implantations of gelatin which were otherwise quite inactive.   Specimens containing induced suckers have been placed in a special class K.   They are perhaps best compared with the rare cases in Urodeles in which an extra balancer has been induced by the implant.

The complete classification of reactions is therefore as follows :—

Class	Reaction
A	Neural tube (positive).
B	Palisade.
B+++ ..	Sunken, many layered palisades (positive).
B++	Palisade much better than controls (positive).
B+	Palisade rather better than controls (? positive).
B—	Control palisade (negative).
C	Rod-like mass of neural tissue (positive).
D	Ectodermal thickening (negative).

E .... Thickening of cuboidal epithelium ["sterol bump," Needham, Waddington, and Needham] (negative).

K .... Sucker [Anura only] (negative).

K' .... Balancer [Urodeles only] (negative ?).

S .... Spina bifida, cannot usually be certainly interpreted, either due to disturbance of gastrulation or to an induced neural plate secondarily connected with that of the host (not included in tables).

I .... No reaction (negative).

The lettering follows the scheme previously described by Needham, Waddington, and Needham, but some of the less important classes mentioned by them have been omitted.

TA

Experiment	Series	Species	No. operated	No. used
Ether ext. Pflüger glycogen	27	Newt	40	15
Ether ext. hydrolysed glycogen	52, 54	Newt	47	9
Ether ext. thymonucleic acid	72	Newt	10	2
,,　　　　,,　　　　..............	80	Discogl.	22	11
Unsaponifiable, Newt	17	Newt	84	10
,,　　　calf liver	33	Newt	24	6
,,　　　pig's liver	95	Axolotl	42	22
Digit. ppt., Windaus, newt	38	Newt	29	11
,,　　　　,,　　　calf	41, 51	Newt	55	4
,,　　　Schönheimer-Dam, calf............	55	Newt	39	9
,,　　　ether extract, glycogen	88	Axolotl	30	6
,,　　　　　,,　　　　..........	82	Discogl.	21	13
Digit. filtrate, Windaus, newt	39	Newt	21	4
,,　　　　,,　　calf	40, 46	Newt	32	9
Fraction X	44, 64	Newt	23	9
Crystals, fraction I, calf	59, 62, 63	Newt	65	16
Digit. ppt. of mother liq. fraction II	60, 65	Newt	45	7
Digit. filtrate of mother liq., fraction III	66	Newt	20	4
B.D.H. cholesterol and residues	47, 67, 68	Newt	41	12
,,　　　　　,,　　　.............	89	Axolotl	20	7
Control albumen	58, 61, 69	Newt	93	32
,,　　　　..........................	87, 96	Axolotl	36	16
,,　　　　..........................	81	Discogl.	21	15
Totals			860	249

The table of results (Table I) mentions also the total numbers of implantations made. It will be apparent that there is great wastage; not much more than a quarter of the implanted embryos are finally classified. This wastage is due partly to the death of the operated embryos in early stages, but the mortality has in general not been particularly high, although in some series the eggs were sensitive and only a small proportion survived. A larger fraction of the unclassified embryos were simply discarded because they showed outwardly no sign of the implant, which was either extruded or, more frequently, buried in the yolky endoderm. Many of these embryos were cultivated till the stage when the presence of any induced neural tissue would have been revealed by the formation of melanophores. In general, only those embryos were

I

I	K	E	D	B—	B+	B++	B+++	C	A	% positive	Remarks
1	—	2	1	—	1	3	1	1	5	67	—
2	1	—	1	5	—	—	—	—	—	0	One balance
—	—	—	—	1	1	—	—	—	—	0	—
2	2	—	7	—	—	—	—	—	—	0	—
1	—	—	1	2	2	4	—	—	—	40	—
—	—	2	1	1	—	—	1	—	1	67	—
5	—	—	6	6	1	1	—	—	3	18	Weak eggs
—	—	1	—	—	1	1	6	1	1	82	—
3	—	—	—	1	—	—	—	—	—	0	—
4	—	1	1	1	2	—	—	—	—	0	—
—	—	2	1	1	1	—	—	—	1	16	Weak eggs
4	6	—	3	—	—	—	—	—	—	0	—
—	—	3	—	1	—	—	—	—	—	0	—
3	—	4	—	2	—	—	—	—	—	0	—
2	—	2	—	4	1	—	—	—	—	0	—
4	—	4	—	5	—	—	—	2	1	18	Mixed stero
2	—	3	2	—	—	—	—	—	—	0	—
1	—	1	2	—	—	—	—	—	—	0	—
2	—	5	5	—	—	—	—	—	—	0	—
1	—	—	—	2	4	—	—	—	—	0	—
14	—	—	7	11	—	—	—	—	—	0	—
—	—	—	9	7	—	—	—	—	—	0	—
3	6	—	6	—	—	—	—	—	—	0	—

sectioned in which there was some external sign of the presence of the implant. The percentages of successful experiments are calculated on the basis of the number of embryos in which the implant has remained in contact with the ectoderm, since it is only in these cases that the implanted substances have had an opportunity to diffuse into the ectoderm and exert their effects.

All the operations were made by one of us (C. H. W.) and it is not possible to explain the different results in the different experiments by reference to technical differences in the handling of the material.

EXPERIMENTS

1—*Controls*

Pure acetone, not containing any dissolved substances, was added slowly to a warm solution of egg-albumen. The coagulum so obtained was implanted into young gastrulæ. As might be expected, there was usually no reaction, but in a few cases the inner layer of ectoderm differentiated into a slight " palisade." The most highly differentiated control palisade is described below (No. C61*a*–13). It defines the lower limit of reactions which might be taken as positive, but actually we have always demanded a considerably higher standard before classifying a specimen in class B++, which is the lowest of our positive classes; specimens intermediate between the best controls and B++ are placed in class B+ and their significance is regarded as doubtful.

The ectoderm of the Axolotl gastrula appears to be more sensitive to the implantation of albumen. It usually reacts by the formation of a very large thickening, often with traces of arrangement into a palisade. These are all counted as B— reactions, and the B+ and B++ classes have not been used for these eggs.

The *Discoglossus* ectoderm, as stated above, often forms induced suckers in response to the implantation of albumen. A typical example is described below.

DESCRIPTION OF SPECIMENS

C61*a*–13. *Control*—The implant consisted of a lump of very hard egg-albumen, coagulated by heating with the addition of acetone, and then immersed in boiling water for a considerable time. The embryo was fixed four days after the operation. The implant has provoked a small thick proliferation of the ectoderm, forming a " control palisade." The figure (fig. 1, Plate 18) shows the best, *i.e.*, most neural, part of this

palisade. The nuclei are arranged on a level parallel to the surface of the implant-mass, but they are not elongated and the cells are not thin and tightly packed, as they are in neural tissue. The cells contain little pigment. The rest of the thickening is made up of cells which show even less of the typical neural characteristics.

C81c–13. *Control*—The embryo was fixed two days after the operation, as a young, already motile tadpole. The implant lies in a large process projecting from the belly. The greater part of the process is made up of thickened ectoderm, but mesenchyme is also present immediately round the implant. The distal end of the implant is differentiated into a sucker. The outer surface of this structure is composed of long columnar cells, heavily pigmented and actively secreting mucus at the time of fixation. Inside this layer are masses of less heavily pigmented cuboidal cells, among which round spaces appear. Pigment tends to be collected around the lining of these spaces which in life were probably filled with mucus (fig. 2, Plate 18). These typical characteristics are rather better developed in another embryo from the same series (C81c–9; fig. 3, Plate 18), where the sucker projects as a knob from the body surface immediately above the implant.

In sum, the specimen of egg-albumen used by us, treated with acetone, is incapable of inducing neural tissue. The slight indications of induction which have been obtained are used to define the lower limit of positive reactions.

Fischer and Wehmeier (1933, *b*), Wehmeier (1934) and Holtfreter (1934, *b*) were also able to demonstrate the comparative inactivity of egg-albumen.

2—*The Ether Solubility of the Evocator*

Fischer and Wehmeier, while admitting with Needham, Waddington, and Needham that active ether soluble substances can be extracted from tissues, assert that the induction of neural plates may be performed by various different substances, among which they name glycogen, adenylic acid, and thymo-nucleic acid. Fischer and Wehmeier found that the activity of their glycogen preparations could be destroyed by long and careful purification through ether and alcohol extraction and dialysis. We have performed the converse experiment, and have shown that active ether soluble substances can be extracted from crude preparations of glycogen, such as were originally used by Fischer and Wehmeier.

Glycogen was prepared by the methods of Pflüger and of Kerly (1930). In the classical method of Pflüger the tissue is digested with strong aqueous potash and the mixture is not neutralized before the precipitation of the

glycogen with alcohol. This has two advantages: firstly, some protein is thrown down and can be spun off before alcohol is added; secondly, precipitation of the glycogen is more complete. Kerly introduced neutralization with trichloracetic acid. In a typical experiment, 160 gm rabbit liver (carrot-fed) was digested for 3 hours on a boiling water-bath with 300 cc of 60% KOH, and then divided into two portions. To the one 400 cc of distilled water were added and the glycogen precipitated by 1200 cc 97% alcohol, bringing the alcohol concentration to the optimum one of 66%. The copious precipitate was washed with 66% alcohol, and dried over calcium chloride. The second portion was neutralized with 40% trichloracetic acid till just acid to bromcresolpurple, and the precipitate left overnight. Then the filtrate was adjusted to the alkaline side of the indicator, and the necessary quantity of alcohol added as before. Washing and drying were also done in the same way.

The other principal method for preparing glycogen consists of coagulating most of the tissue protein by boiling, followed by the removal of the remainder by trichloracetic acid, and the precipitation of the glycogen in the filtrate by alcohol. We have had in our possession samples prepared by two variants of this method, F. G. Young (private communication) and H. Lund (private communication), but we have not as yet studied the evocation properties of these specimens. We hope to discuss this and similar questions in a later communication.

Extraction of the glycogen was carried out for 50 hours in a Soxhlet apparatus with ether.

It was thought that the evocator substance might more readily be eluted from the glycogen if the latter were hydrolysed. Samples were therefore hydrolysed for 6 hours with $2 \cdot 2\%$ HCl on a boiling water-bath, the solution (which had completely lost its opalescence) neutralized, evaporated nearly to dryness, and solidified with pure anhydrous sodium sulphate. The dry pulverized mass was then extracted for 50 hours with ether in a Soxhlet apparatus.

The literature appears to contain no information about the adsorption of ether-soluble substances on glycogen. According to most authors (e.g., McDowell, 1927; Petree and Alsberg, 1929; Sahyun and Alsberg, 1930; Taylor and McBride, 1931; and Bell and Young, 1934) it is possible to prepare glycogen free from ash, but not from phosphorus. Doubt still exists whether this is attached within the molecule itself, or to some accompanying nitrogenous substance which cannot be separated from it. Willstätter and Rohdewald (1934) have recently investigated the formation and properties of glycogen-protein adsorption complexes in liver and muscle. Even this work, however, throws no direct light

on the loose combination between glycogen and ether-soluble substances envisaged here.   Taylor and Sherman (1933) found fatty acids adsorbed on starch almost impossible to remove with solvents, the substances only being set free after long continued hydrolysis.

Ether extracts have also been prepared from a specimen of thymonucleic acid (B.D.H.).   These have been tested in a small number of experiments on *Triton* and *Discoglossus*.   No positive results were obtained, as is not surprising considering the insensitiveness of the *Discoglossus* ectoderm.

Descriptions of Specimens

C27b–5.   Ether Extract of Pflüger-glycogen—The embryo was fixed in the tail-bud stage, 4 days after the operation, when it had a sharp knob-like swelling, coloured brown, just above the implant.   The sections show that this knob contains a good induced neural tube, with a little mesenchyme, but no definite mesodermal organs (fig. 4, Plate 18).   The neural tissue is rather irregularly shaped, but may perhaps represent some part of an embryonic brain; it lies in the anterior part of the host's belly. The neural tube is well developed only over the anterior part of the implant; posteriorly the lumen dies out, and the rod of neural tissue thus formed becomes thinner and flatter and passes into a B++ palisade.

C27b–23.   Ether Extract of Pflüger-glycogen—The embryo was fixed 3 days after the operation.   The sections show that the small pigmented knob which could be seen on its surface contains an induced neural tube again without any accompanying induced mesodermal organs, fig. 5, Plate 18.

Result—Glycogen prepared from rabbit liver by the Pflüger method contains an ether soluble impurity which is able to evocate.   Probably the whole of the activity of the crude glycogen is due to the admixture of this substance.   As will be shown later, the active substance is also present in the digitonin-precipitate prepared from the ether extract of glycogen.

3—The Presence of the Evocator in various Fractions of the Ether Extract

Ether extracts were prepared from the whole bodies of adult *Triton tæniatus,* and from calf liver.   The tissue was ground up with anhydrous sodium sulphate, and then left some days in a desiccator.   The dry and finely pulverized mass was then extracted with ether for 25 hours in a

Soxhlet apparatus. After removal of the ether by distillation, the extracted material was obtained in the form of a dark yellow oil.

This extracted substance was not itself implanted, since the work of Needham, Waddington, and Needham had already shown that it might be expected to contain the evocator. Further fractions were therefore prepared.

Saponification

The extracted oil was weighed, and boiled under a reflux condenser with 25 cc (in some cases 50 cc) of 2N alcoholic potash (97%) for 2 hours. In the previous experiments of Needham, Waddington, and Needham the unsaponifiable fraction had been found to be alkaline and therefore lethal when implanted into the test embryos. We therefore added aqueous hydrochloric acid in quantities sufficient to bring the potash to about N/10. In calculating the quantity required, it was assumed that half the extracted oil consisted of fats with an average molecular weight of 800 and half of fatty acids with an average molecular weight of 300, in order to be on the safe side. After addition of the acid, the unsaponifiable fraction was removed by 2 hours continuous extraction with petrolether, in a Dakin apparatus. It was made sure that the reaction remained definitely alkaline.

The unsaponifiable fraction of the extract from adult newts was tested in experiments C17a and C18a, and that from the calves' liver in experiments C33a and C33b. The saponifiable fraction was not tested. The test embryos reacted to the implants mainly by the formation of palisades considerably better than the control palisades. In one case, a palisade passed directly into a thin walled induced neural tube.

Experiments have also been made to test the activity of the total unsaponifiable material extracted from pig's liver, a specimen of which was obtained from Professor Channon. The results are rather unsatisfactory owing to the fact that the experiments were made with a batch of very weakly Axolotl eggs, so that the embryos could not be kept alive for more than a day or two. Positive results were, however, obtained.

DESCRIPTION OF SPECIMENS

C33a–3. Unsaponifiable Fraction of Ether Extract of Calf Liver—The embryo was fixed 5 days after the operation, in the stage of the first muscular contractions. The surface of the implant which is in contact with the ectoderm is, for most of its length, wrapped in a one layered palisade, in which the cells and nuclei are very thin, elongated, and closely packed (B+ +). At one end this passes into a thick mass of neural

cells, at the other into a short thin walled neural tube, the lumen of which is partly filled by implant material, fig. 6, Plate 18.

C17a–43. Unsaponifiable Fraction of Ether Extract of Whole Newts— The embryo was fixed in the old tail-bud stage, 4 days after the operation. The implant and the accompanying ectodermal thickening lie just anterior to the tail on the ventral surface. The thickening consists of an outer part made up of cuboidal cells, not arranged in any particular order (D type), and an inner part, of several layers of elongated, parallel cells, with oval darkly staining close packed nuclei (B++ palisade).

Result—The unsaponifiable portion of the ether extracts contains an evocating substance.

Digitonin Precipitation

The unsaponifiable fractions were separated into two parts by digitonin precipitation. The first method employed was that described by Windaus (1910). The petrol ether was removed from the unsaponifiable material by distillation, and the material dissolved in a small quantity of warm 97% alcohol. To this a solution of 1 or 2% digitonin (dissolved in 97% alcohol) was added drop by drop until no further precipitate appeared. The precipitate was filtered off after standing on ice. The completeness of the precipitation was tested by the addition of 1 cc of digitonin to the filtrate, and a second filtration was made if necessary. After the addition of its own volume of distilled water, the filtrate was extracted several times with petrol ether by shaking in an extraction funnel, and the last traces of digitonin removed from the petrol ether by washing with distilled water. The mixed digitonides were washed on the filter paper with ice-cold ether, and then dried in a desiccator. In order to remove the digitonin the precipitate was boiled in xylene for 8 hours under a reflux condenser in an oil bath. The xylene solution was filtered off, the xylene removed by distillation *in vacuo* and the material thus obtained dissolved in petrol ether.

These preparations were tested in the experiments C38b (precipitate) and C39b (filtrate) from the newt unsaponifiable, and C41a, C41b, C41e, C51a (precipitate) and C40b, C40e, C46a (filtrate) from the calf liver unsaponifiable.

The above method was not entirely satisfactory, as we found that during the boiling with xylene a substance appeared which was insoluble in alcohol and which had contaminated the digitonide-precipitate. It is possible that our digitonin was not quite pure. The substance, however, could not be obtained from the digitonin by ether extraction; it appeared

only after boiling with xylene. This substance was tested as " fraction *x* " in experiments C44*a* and C64*a*.

On the advice of Dr. Rosenheim, we adopted a modification of Windaus's method proposed by Schönheimer and Dam (1933). In this method the digitonin precipitate is dissolved in a very small quantity of anhydrous pyridine, and the digitonin removed by precipitation with an excess of ether. The sterols then remain in solution. After filtration, the pyridin was distilled off *in vacuo*, and the material so obtained dissolved in petrol ether. The precipitate prepared in this way was tested in experiment C55*a* (calf liver).

Crystallization

It was found that, if the unsaponifiable material was allowed to come down from alcohol solution in the cold before the digitonin precipitation was performed, white crystals were obtained which probably consisted largely of cholesterol (positive Liebermann-Burchardt reaction). It was hoped that in this way it might be possible to separate the main mass of the cholesterol from the evocator, but this hope was not realized. After removal of the petrol ether, the unsaponifiable material was taken up in 5–10 cc of absolute alcohol with the addition of 5 cc of distilled water, and the solution left 4–8 hours in the ice chest. The crystals which had formed were removed by filtration at the pump, washed several times with ice cold alcohol of the same concentration and dried (fraction I). A digitonin precipitation was then done, by the Schönheimer-Dam method, on the mother liquor, giving the fractions II (precipitate) and III (filtrate). The material used was derived from calf liver. The fractions were tested in the experiments C59*a*, C59*b*, C62*a*, C63*a* (fraction I, crystals), and C60*a*, C65*a* (fraction II, digitonin precipitate of mother liquor), and C66*a* (fraction III, final filtrate).

We also tested " pure " cholesterol of British Drug Houses (experiments C47*a*, C68*a*) and the ultimate residues left after the preparation of cholesterol which were very kindly supplied to us by that firm (experiment C67*a*).

Digitonin Precipitation of Glycogen Extract

A digitonin precipitate was also prepared, by the Schönheimer-Dam method, from the ether extract of Pflüger glycogen, which had been proved to be strongly active. This preparation was only made at the end of the egg-laying season. It was used on a few *Triton* gastrulæ, with no result, and later, in October, on some Axolotl embryos. The latter

were very weakly, and the mortality was high and development bad. However, one positive result was obtained.

DESCRIPTION OF SPECIMENS

C38b–5. Digitonin Precipitate of Newt Unsaponifiable—Fixed 3 days after the operation. The implant has induced the formation of a thick strand of columnar epithelium. This originates from the inner layer of the ectoderm, but has sunk away from the outer layer, and at each side abruptly thins out to a slender film of tissue connecting with the unmodified inner ectodermal layer, which is in close contact with the outer layer in the usual way. The whole structure can be shortly described as a " sunk abrupt " palisade (type B+++). The thickened strand of tissue might at first sight be taken to be formed from the mesoderm, which sometimes collects in a slightly similar way round the implants, but examination shows that the mesoderm is interrupted by the implanted mass and that the " sunk abrupt " tissue is really ectodermal. The tissue is for most of its length closely adherent to the surface of the implant. The section shown passes through one end of the albumen, and here, where the mechanical conditions are suitable, the palisade rolls up into a tube, fig. 7, Plate 18.

C38b–24. Digitonin Precipitate of Newt Unsaponifiable—Fixed 3 days after operation, in stage of first muscular contractions. The figure shows a typical many layered " sunk abrupt " palisade. It passes anteriorly into a one layered thickening which is continuous with the thickening of the inner layer of the ectoderm just below the host's head, fig. 8, Plate 18.

C39b–1. Filtrate from Digitonin Precipitate used in Experiment C38b above—Embryo fixed 3 days after operation, infected in the head region. There is a large ectodermal thickening, but the cells are quite disordered, and are more or less cuboidal in shape with round nuclei. Both layers of the ectoderm seem to be involved (class E), fig. 9, Plate 18.

C88d–27. Digitonin Precipitate of Ether Extract of Glycogen—The experiment was made with Axolotl eggs obtained in the early autumn. The eggs were very weakly, did not develop well when left alone, and showed an enormous mortality when operated. The embryo was fixed 3 days after operation, in a young tail-bud stage. The host neural tube is thin walled, and the embryo clearly unhealthy. Lying over the centre of the implant is a small induced neural tube, still connected with the

ectoderm. The cells of its walls are still rounded, and the nuclei are not particularly elongated.

C59b–1. *Crystallization* 1, *Fraction* 1; *Crystals from Calf Liver Unsaponifiable before Digitonin Precipitation*—The embryo was fixed 4 days after operation, in the elongated tail-bud stage. The implant is in the anterior belly, and has induced a very large ectodermal thickening, which is mostly made up of cells of the cuboidal, " sterol-thickening " type (class E). But in one place there is a rod of neural tissue, recognizable by its narrower, more closely packed and more deeply staining nuclei. In some sections there are suggestions that this mass is really a tube, but the sections do not cut it quite transversely, and it is difficult to be sure if there is a narrow lumen or not, fig. 10, Plate 18.

Result—An evocating substance was clearly present in the digitonin precipitate prepared from newt material, whereas it was absent or weak in the filtrate from this same preparation. The substance may therefore be taken to be precipitable with digitonin. It is not clear why negative results were obtained with the digitonin precipitates from calf liver; they may depend on the small numbers of successful operations. Similarly it is very possible that further work will show that the filtrates contain small quantities of the active substance, since the separation may not be complete, but the evidence of experiments C38 and C39 strongly suggests that the main amount of the substance is included in the. precipitate. Confirmatory evidence is brought by the experiment C88*d*–27 (digitonin precipitate of ether extract of glycogen in Axolotl), where an induction has been obtained even in a thoroughly unhealthy host embryo.

It is also clear that the active substance comes out with cholesterol in fraction 1 of the crystallization. The tests of B.D.H. cholesterol have not yet been brought to a satisfactory conclusion, but there is some evidence, from experiment C89*d*, that it is weakly active. These operations again were made on the unhealthy Axolotl eggs, and the substance may not have been able to show its full effects. Further tests of this substance will be made next year as soon as eggs become available.

Discussion

It has been shown that the evocating substance which is extracted by ether from adult tissues is unsaponifiable, precipitable with digitonin, and separable with the cholesterol from the alcoholic solution of the crude unsaponifiable matter. These facts strongly suggest that the substance is a sterol.

One further suggestion emerges from the facts described in the body of the paper. It is noticeable that the ether extract of Pflüger glycogen was the most active extract which we have prepared, whereas the extracts of the Kerly glycogen and of the hydrolysed glycogen were comparatively inactive. If this is a real phenomenon, and not merely due to chance or the conditions of emulsification of the substance, it suggests that the evocator substance is destroyed by prolonged boiling with acid. Holtfreter (1934, *a*) finds the activity of tissue probably reduced after 20 hours treatment with cold 20% hydrochloric acid.

Proceeding on the assumption that different authors are describing the properties of the same substance, we may attempt to summarize the information not contained in the foregoing parts of this paper.

Several authors have attacked the problem of the solubility of the evocator. Fischer and Wehmeier (1934) confirmed Needham, Waddington and Needham's discovery that active ether extracts can be prepared, and also demonstrated the activity of alcohol and acetone extracts. There is, therefore, no doubt that the evocator is soluble in these solvents. The same authors have demonstrated a weaker evocating power in crude extracts of various tissues made with boiling water, but it seems very doubtful if one should conclude from this that the evocator is soluble in hot water as it is likely that such extracts would contain fats in an emulsified form. Holtfreter (1934, *a, b*) proved the presence of the evocator in the yolk-free fractions of the centrifugate of crushed tissues, as did Needham, Waddington and Needham contemporaneously. In Holtfreter's experiments the watery protein-rich fractions gave better results than the uppermost fatty fraction, but the number of implantations was not large and the separation of substances obtained by centrifugation is not complete enough to enable definite conclusions to be drawn, although the facts suggest that, if the evocator is of the sterollike nature suggested, it may be attached to some protein or other substance rather than free. Barth (1934) has also obtained inductions with ether extracts, prepared in his case from brain, and claims that it is the cephalin fraction which is active, but he admits that the separation from the sterol fraction is probably incomplete; it is perhaps unfortunate that he regarded his implantation medium, namely kaolin, as sufficiently inactive to obviate the necessity for controls, although it is known to lead to inflammatory conditions when placed under the skin of adult animals.

This exhausts the results of the testing of tissue extracts. Most of the work on the solubility of the evocator has been carried out by testing the activity of the residue left after extraction by the solvent. Fischer

and Wehmeier (1934) and Holtfreter (1934, *a*), with a variety of organic solvents, find that the activity of the residues is considerable, often very little less than that of the unextracted material. Fischer and Wehmeier therefore speak of the " Schwerlöslichkeit " of the evocator in organic solvents. Barth also gives a list purporting to show the solubility of the evocator in various solvents, collected from Fischer and Wehmeier's and Holtfreter's results. As we have previously pointed out, these experiments really give practically no information about the solubility of the evocator; they concern its extractability, which is quite 'a different thing. Thus Fischer and Wehmeier themselves, in the same paper in which they speak of " Schwerlöslichkeit," point out that the evocator is very easily obtained in ether, alcohol and acetone extracts. One can only say, therefore, that the testing of the residues has given no information about the solubility of the evocator, but has shown that it is only with very great difficulty completely extracted from tissue.

An isolated piece of information is Holtfreter's demonstration that the evocator is destroyed at temperatures above 120° C in the dry state and also by prolonged boiling.

SUMMARY

An evocator, *i.e.*, a substance capable of causing the ectoderm of the amphibian gastrula (*Triton* spp., Axolotl) to differentiate into neural tissue, has been obtained in ether extracts of whole newt bodies and of mammalian liver. The active substance is present in the unsaponifiable fraction, and in the part of that fraction precipitable with digitonin. It comes out with the cholesterol if the unsaponifiable fraction is allowed to crystallize from alcohol in the cold. It is suggested that the substance is of a sterol-like nature.

An active ether soluble substance, which is also precipitable with digitonin, has been isolated from crude preparations of glycogen. It is suggested that the whole of the evocating activity of glycogen, which has been reported by other workers, may be due to the admixture of this substance.

The ectoderm which is brought into contact with these substances shows a graded series of reactions which suggests that the quantity of the substance available plays a part in influencing the differentiation which the ectoderm undergoes.

In experiments with the eggs of the Anuran *Discoglossus pictus* it was found that stimuli which were probably purely mechanical could cause the formation of a sucker.

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DESCRIPTION OF PLATE 18

I.th.   Induced thickening.   *I.s.*   Induced sucker.
I.n.t.   Induced neural tube.   *Imp.*   Implant.

FIG. 1—C61a–13. Control implant of albumen, showing very slight neuralization of ectoderm.

FIG. 2—C81c–13. Control implant of albumen into *Discoglossus*. Sucker-like induced thickening.

FIG. 3—C81c–9. Control implant of albumen into *Discoglossus*. Note induced sucker.

FIG. 4—C27b–5. Ether extract of Pflüger glycogen.

FIG. 5—C27b–23. Ether extract of Pflüger glycogen.

FIG. 6—C33a–3. Unsaponifiable matter from calf liver. B++ palisade rolling up into a neural tube.

FIG. 7—C38b–5. Digitonin precipitate, newt, unsaponifiable. B+++ palisade rolling up into a tube.

FIG. 8—C38b–24. Digitonin precipitate, newt, unsaponifiable. B+++ palisade.

FIG. 9—C39b–1. Digitonin filtrate, newt, unsaponifiable. E type thickening.

FIG. 10—C59b–1. Crystallization 1, fraction 1, calf liver, unsaponifiable. E type thickening with rod of neural tissue.

Studies on the Nature of the Amphibian Organization Centre*

II—Induction by Synthetic Polycyclic Hydrocarbons

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[PLATE 19]

INTRODUCTION

The connection between the growth and differentiation, or dedifferentiation, of tumour tissue and the normal processes of growth and development which take place during ontogeny is obscure; the problem has hardly yet been studied from a modern point of view. Some connection

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* A preliminary communication of the results reported in this paper has been made (Waddington, Needham, Nowinski, Needham and Lemberg, 1934).

there must be. It is clear that one of the questions which must be answered about tumours is the question of how the tissue has escaped from the control of those agents which govern the ordinary growth of the body. In animals in which regeneration occurs it is known that the regenerative growth is controlled by agents which in many respects behave like the organization centres on which the earlier embryonic development is so largely dependent. Even in other animals the normal repair-growth of the body is probably dominated by factors of the same general type. Various lines of work are now being tested to see whether any connection can be found between the phenomena of tumour formation and regeneration or other aspects of the activity of organization centres.

The accumulation of evidence suggesting that the active principle of the amphibian organization centre, the evocator, is an unsaponifiable ether-soluble substance, suggested that it might be worth investigating whether any of the known members of this biologically active group of substances could produce the effects normally associated with the evocator. For the preliminary investigation some of the carcinogenic and œstrogenic substances recently prepared by Cook (1933, 1934) were chosen. Their activity was tested by the methods described in the preceding paper, namely, by the implantation into the cavity of young amphibian gastrulæ of small pieces of coagulated egg-albumen which contained, in an emulsified form, the substance to be tested.

The Experiments

The coagula were prepared by one of us (D. M. N.) in the way described in the preceding paper. The substances were used in concentrations of about 2 mg per cc, but the concentrations were not accurately measured. The following substances were tested: 1 : 9-dimethylphenanthrene, 9 : 10-dihydroxy-9 : 10-di-*n*-butyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene, 1 : 2 : 5 : 6-dibenzanthracene, 5 : 6-*cyclo*penteno-1 : 2-benzanthracene, 1 : 2-benzpyrene, dehydronorcholene, methyl-cholanthrene. The first two of these have given clear evidence of their ability to evocate the formation of secondary neural tubes, and there is evidence of a weaker activity of the third substance; the results with the others have so far been entirely negative. A list of results is given in Table I. The other biological activities of these substances have been described by Cook, Dodds, and others (see summary of Dodds (1934), and also Cook and Haslewood (1934)).

C32b–1.   9 : 10-dihydroxy-9 : 10-di-*n*-butyl-9 : 10-dihydro-1 : 2 : 5 : 6-
dibenzanthracene.

The embryo was fixed in a tail-bud stage, 3 days after the operation. Lying against the implant-mass is an irregularly shaped induced neural tube, passing into a solid rod of neural tissue, which projects as a small process from the anterior belly of the host, fig. 1, Plate 19.

C49b–9.   1 : 9-dimethylphenanthrene.

On the day after the operation the embryo could be seen to have a process with a pigmented groove (induced neural plate) lying above the implant.   It was fixed 2 days after the operation, and the sections show a typical induced neural tube, accompanied by a little loose mesenchyme, but no somites or other mesodermal organs, fig. 2, Plate 19.

C79c–9.   1 : 9-dimethylphenanthrene.

The substance was implanted into a *Discoglossus* gastrula, which was fixed 2 days later in a young tadpole stage.   The implant lies against the belly wall and has induced the formation of a large swelling of thickened ectoderm which shows no signs of differentiating into a sucker.   In the middle of the thickened ectoderm is a small tube of heavily pigmented cells, fig. 3, Plate 19.   The pigment is distributed evenly throughout the whole of each cell, and the nuclei are smaller and more darkly staining than those of the other ectoderm cells.   These are characteristics of the neural tissue in this species, and there can be no doubt that the tube represents an induced neural tube, although in its gross form it is somewhat atypical. Other very similar neural tubes have been induced in *Discoglossus* by implants of the blastopore lip.

C84c–11.   1 : 2 : 5 : 6-dibenzanthracene.

The *Discoglossus* embryo was fixed 2 days after the operation.   A large process could be seen projecting outwards and forwards from the middle of the belly.   This projection does not lie immediately above the implant but a little posterior to it ; probably the implant has shifted during the course of development.   Within the projection, sections show the presence of a small neural tube lying against the ectoderm in the posterior part of the structure where it joins the body of the host, fig. 4, Plate 19. The anterior part of the projection is filled with mesenchyme and with a thick mass of tissue, probably of mesodermal origin, which seems to show some signs of metameric segmentation ;   its exact nature cannot be determined with certainty, fig. 5, Plate 19.
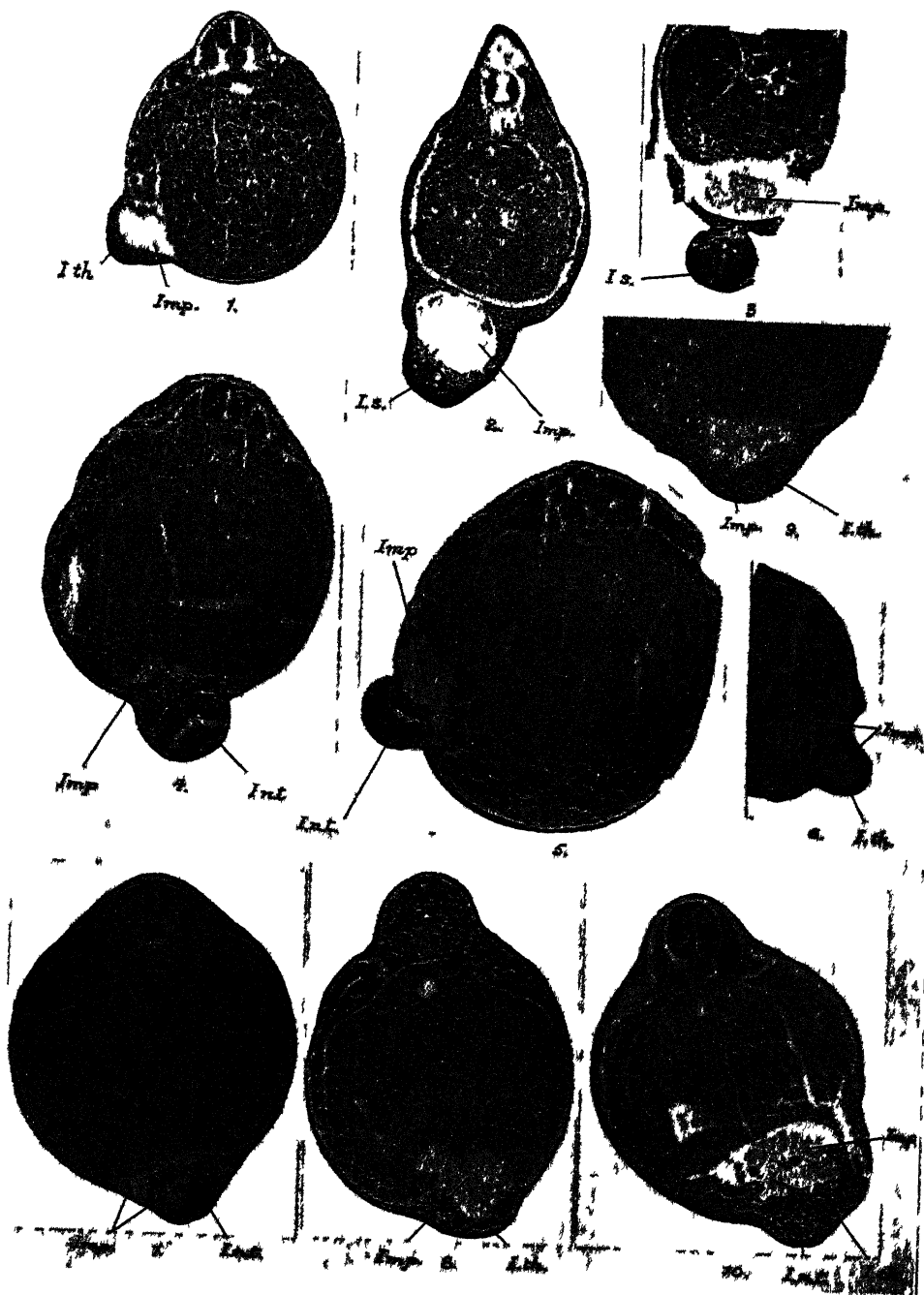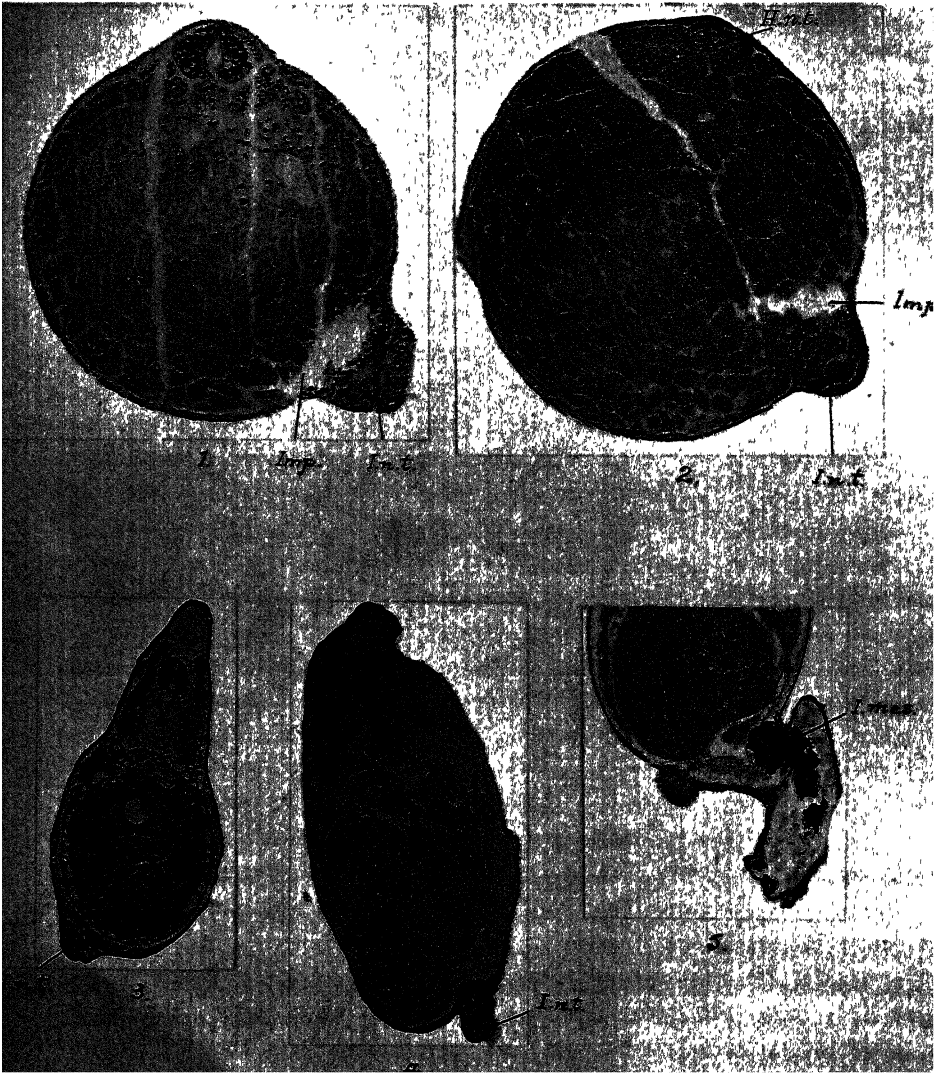
Results—The experiments described above, and those recorded in Table I, show that evocations can be performed by 1:9-dimethyl-phenanthrene, 9:10-dihydroxy-9:10-di-*n*-butyl-9:10-dihydro-1:2:5:6-dibenzanthracene and 1:2:5:6-dibenzanthracene. The evidence is quite clear in the case of the first two substances, but not so definite for 1:2:5:6-dibenzanthracene, which only produced weak effects in newt embryos and one induction in *Discoglossus*. With the other substances tested only negative results were obtained.

DISCUSSION

The results of the above experiments, which demonstrate that a capacity to evocate is possessed by synthetic hydrocarbons of the phenanthrene group, confirm in a most striking manner the conclusions which have been drawn in the preceding paper. These hydrocarbons are also ether-soluble and unsaponifiable, like the substances of which the isolation and testing was there described. The present experiments, in fact, add one important fact to the series of observations from which it was concluded that the naturally occurring evocator is an ether-soluble, unsaponifiable substance. The evidence derived from the purification of extracts of animal tissues is always open to the objection that active impurities of a totally different nature may be associated with the fractions which are assumed to be tested in the experiments. One instance in which this objection seems to be justified is the case of glycogen. Again, it is true that the digitonin precipitates are not unlikely to be contaminated by other unsaponifiable substances. But the synthetic hydrocarbons, since they are not of animal origin can scarcely be supposed to be contaminated by unallied active substances. If they are contaminated at all, it can only be with minute traces of nearly allied substances of similar general properties. Their activity then is very strong evidence that certain ether-soluble, unsaponifiable substances actually do possess the power of evocation.

It is unlikely that the activity of the highly purified potent preparations which have been tested was due to admixture of some other active substance of similar chemical nature; it is much more probable that it is a property of the substances themselves. If this is so, we must suppose that evocation can be performed by more than one substance, namely, by 1:9-dimethylphenanthrene, by 9:10-dihydroxy-9:10-di-*n*-butyl-9:10-di-hydro-1:2:5:6-dibenzanthracene, by 1:2:5:6-dibenzanthracene, and by the natural evocator, if it should be different from these. The evidence for the non-specificity of the evocator which is presented by the experi-

Substance	Activity	Series	Species	No. operated	No. used
1 : 9-dimethylphenanthrene	œstrogenic	49	Newt	18	5
,, ...	,,	79	Discog.	29	17
,, ...	,,	85, 101	Axolotl	55	2
9 : 10-dihydroxy-9 : 10-dihydro-9 : 10-di-	œstrogenic	32	Newt	17	6
n-butyl 1 : 2 : 5 : 6-dibenzanthracene	,,	78	Discog.	25	17
,, ,,	,,	86, 97	Axolotl	38	5
1 : 2 : 5 : 6-dibenzanthracene	carcinogenic	48	Newt	23	5
,, ...	,,	84	Discog.	22	10
1 : 2-benzpyrene	carcinogenic and œstrogenic	20, 57	Newt	53	16
5 : 6-cyclopenteno 1 : 2-benzanthracene	carcinogenic and œstrogenic	34, 35	Newt	23	4
Dehydronorcholene	carcinogenic	70	Newt	10	3
Methylcholanthrene	carcinogenic	71	Newt	15	5
Control albumen	—	58, 61, 69	Newt	93	32
,, ...	—	87, 96	Axolotl	36	16
,, ...	—	81	Discog.	21	15
Totals				478	158

ments described above seems more convincing than that adduced by Fischer and Wehmeier (1933) and discussed in the preceding paper.

The stimulation of specific reaction systems by non-specific chemical substances has become well known through the recent work of Dodds, Kennaway, Cook, and their collaborators. They have not only been able to prepare a series of substances which can produce cancerous growths when applied to the skin, and another series which produce the symptoms of œstrus in the way characteristically called forth by the sex-hormone œstrin, but have also shown that these two groups of substances overlap, and that some of the carcinogenic substances are also œstrogenic. The œstrogenic group again overlaps with the group of the sterols, bile acids, and vitamin D, some derivatives of which have a

1

I	K	E	D	B—	B+	B++	B+++	C	A	% posi- tive	Remarks
—	—	—	1	—	1	1	—	—	2	60	—
1	9	—	6	—	—	—	—	—	1	6	—
—	—	—	—	—	—	—	—	—	? 2	—	One other noted alive. Weak eggs
—	—	1	—	—	—	1	2	1	1	82	—
—	9	—	8	—	—	—	—	—	—	0	—
—	—	—	1	2	1	—	—	—	? 1	—	Weak eggs
—	—	2	—	—	1	2	— ·	—	—	40	Weak effect
1	3	—	5	—	—	—	— -	—	1	10	—
3	—	8	2	3	—	—	—	—	—	0	—
— ·	—	4	—	—	—	—	—	—	—	0	—
1	—	—	1	1	—	—	—	— ·	—	0	—
—	—	2	3	—	—	—	—	—	—	0	—
14	—	—	7	11	—	—	—	—	—	0	—
—	—	—	9	7	—	—	—	—	—	0	—
3	6	—	6	—	—	—	—	—	—	0	—

definite œstrogenic activity. We are now able to add a further type of activity to the series of overlapping functions; two œstrogenic substances are evocators. Further work is necessary to show how widely the evocating capacity is spread; 1 : 2 : 5 : 6-dibenzanthracene, which is a powerfully carcinogenic but not an œstrogenic substance, is also weakly evocating, but none of the other carcinogenic substances has as yet shown any activity.

It is as yet too early to speculate on the mechanism by which these different substances can perform similar functions. Although most of them belong to the group of phenanthrene derivatives, the differences between them are not small, and it is very difficult to guess why one particular set should be carcinogenic, another œstrogenic, another

evocating and so on.   Our conception of the chemical basis of stimulatory activity is undergoing a rapid revision. Dodds has metaphorically spoken of these synthetic substances as skeleton keys, which can unlock several doors. But even this idea can scarcely apply to auxin, the plant hormone which stimulates cell extension in seedlings (see Went, 1928; Kögl, 1931). Naturally occurring auxin, according to Kögl and Erxleben (1934), consists of a pentacyclic carbon ring with one double bond and three side-chains, the two shorter ones being branched. But the auxin found in human urine proved to be identical with β-indolacetic acid (Kögl, Haagen-Smidt, and Erxleben, 1934). Here the skeleton key is so unlike the householder's latchkey that one wonders whether the house has not been entered through the backdoor, or, in an even more unorthodox manner, through a window.

Summary

Certain synthetic hydrocarbons have been implanted into young amphibian gastrulæ. Inductions of neural tissue have been performed by 1:9-dimethylphenanthrene, 9:10-dihydroxy-9:10-di-n-butyl-9:10-dihydro-1:2:5:6-dibenzanthracene, and (less strongly) 1:2:5:6-dibenzanthracene. The first two of these are œstrogenic and the third carcinogenic. There is, therefore, probably a group of evocating substances which overlaps with the group of œstrogenic and carcinogenic substances.

Since the active substances were prepared synthetically, they cannot have been contaminated with active impurities of animal origin, and there can be little doubt that the activity found was due to the substances named or other substances of very similar nature associated with them in the synthetic product.

This provides the first satisfactory evidence that more than one substance is capable of evocating.

The evidence produced supports the suggestion that the naturally occurring evocator is a sterol-like substance.

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DESCRIPTION OF PLATE 19

I.n.t., induced neural tube.   *H.n.t.*, host neural tube.
I.mes., induced mesoderm.

FIG. 1—C32b–1. 9 : 10-dihydroxy-9 : 10-di-*n*-butyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenz-anthracene.

FIG. 2—C49b–2. 1 : 9-dimethylphenanthrene.

FIG. 3—C79c–9. 1 : 9-dimethylphenanthrene. (*Discoglossus.*)

FIG. 4—C84c–11. 1 : 2 : 5 : 6-dibenzanthracene. *Discoglossus.* Small induced neural tube.

FIG. 5—Same specimen. Tail-like outgrowth.

The Production of Cancer by Pure Hydrocarbons— Part III

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(From the Research Institute of The Cancer Hospital (Free), London)

[Plates 20 and 21]

The carcinogenic properties of 1 : 2 : 5 : 6-dibenzanthracene, 5 : 6-*cyclo*-penteno-1 : 2-benzanthracene and some closely related compounds have already been described (Part I, Cook, Hieger, Kennaway and Mayneord, 1932; Part II, Cook, 1932, *a*; Burrows, Hieger and Kennaway, 1932; Burrows, 1932 and 1933; Barry and Cook, 1934). The present communication records the results of a systematic examination for carcinogenic activity of tetracyclic and pentacyclic aromatic hydrocarbons, and also deals with the extension of this work along lines suggested by the results obtained.

Technique

(1) Tests for the production of cancer of the skin were carried out by applying the substance in solution to the interscapular region of mice twice weekly. In the great majority of cases the compound was applied in benzene (0·3 gm in 100 cc), whether this gave a saturated solution or not. The advantages and disadvantages of this method have been discussed in an earlier paper (Part I). Any other concentrations or solvents used are stated in the tables below. The results are shown in Tables I–IX.

(2) Tests for the production of sarcoma were made by injecting the compound *sub cutem* in a fatty medium, generally lard; details are given in the various sections on next page.

Tetracyclic Compounds

Tests have now been completed (Tables I and III) with the six possible hydrocarbons which have molecules consisting of four condensed aromatic rings; the formulæ of these are given below. Evidence of any considerable cancer-producing properties was obtained in only one case, namely, with 3 : 4-benzphenanthrene. This compound is of particular interest, in that it is the simplest hydrocarbon yet shown to have pronounced

carcinogenic activity (7 epitheliomas and 5 papillomas in 20 mice, fig. 1). Moreover, it was the only one of six compounds under discussion which was not already known, and it is the only actively carcinogenic compound yet encountered which is not derived from, or related to, 1:2-benzanthracene. This latter circumstance clearly indicates that it is undesirable to confine the search for cancer-producing activity to compounds which contain the ring system of 1:2-benzanthracene.
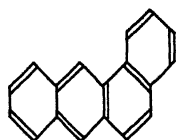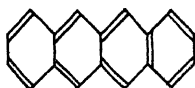
1:2-Benzanthracene   2:3-Benzanthracene   Chrysene
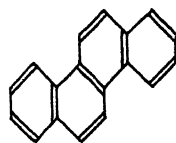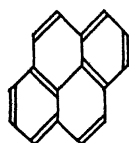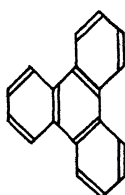
Pyrene   Triphenylene   3:4-Benzphenanthrene
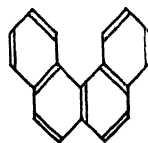
Entirely negative results were obtained with triphenylene and 2:3-benzanthracene (naphthacene), and also with 9:10-dihydronaphthacene. In order to make more complete the comparison of the two benzanthracene ring systems, 6-*iso*propyl-2:3-benzanthracene was also tested, with negative results. The analogous 6-*iso*propyl-1:2-benzanthracene has moderately potent cancer-producing properties (Cook, 1932, *a*; Table III).

Evidence of very slight carcinogenic power was obtained with chrysene, pyrene and 1:2-benzanthracene. 1:2-benzanthracene when applied to the skin gave only one epithelioma in 80 mice. This compound was injected also *sub cutem* (4 mg in 1 cc lard) into 10 mice, of which six were alive after 1 year, when the experiment was discontinued, the result having been negative. An extensive investigation of the action of chrysene has yielded 3 papillomas and 1 epithelioma in 290 mice (Table I). The chrysene used in these experiments, although satisfying the usual criteria of purity (*cf.* Barry and Cook, 1934), was presumably obtained from coal tar, and it is impossible to say that these four tumours were actually produced by chrysene and not by a trace of a persistent impurity, for in our experience it is a particularly difficult task to remove traces of certain other hydrocarbons from chrysene. Hence the question whether

TABLE I—TETRACYCLIC COMPOUNDS NOT RELATED TO 1 : 2-BENZANTHRACENE

Compound	Method of preparation	Mice				Tumours	
		Initial number	Alive after		Day of death of last mouse	Epithelioma	Papilloma
			6 months	1 year			
Naphthacene (2:3-benzanthracene) ..	Fieser (1931)	10	10	8	508	0	0
β-isoPropylnaphthacene (6-isoPropyl-2:3-benzanthracene)	Cook (1934)	10	10	10	819	0	0
9:10-Dihydronaphthacene)	Fieser (1931)	10	10	7	466	0	0
Triphenylene	Diels and Karstens (1927)	10	10	4	548	0	0
Pyrene—							
(1) Crude, from coal-tar (1% in benzene)	—	5	3	3	634	0	1
(2) Sample (1) above after purification through picrate	—	20	15	8	600	0	1
(3) "Pure pyrene" supplied by Gesellschaft für Teerverwertung ..	—	20	18	5	717	0	1 and ?1
†2-Methyl pyrene ..	Cook and Hewett (1934) ..	10	10	5	3 alive day 490*	0	0
unsym. Hexahydropyrene	Cook, Hewett and Hieger (1933)	10	8	1	461	0	0
sym. Hexahydropyrene	Cook, Hewett and Hieger (1933)	10	9	7	664	0	0
3:4-Benzphenanthrene	Cook (1931, c)	20	18	14	676	7	5
Chrysene—							
(1) Sample A (kindly given by Mr. Gordon Adam) 0·3% in benzene	—	50	30	24	775	0	2
(2) Sample A, 7·5% in comml. oleic acid	—	100	77	44	797	0	0

			290					
(3) Sample B, from Rütgerswerke A-G, 0·3% in benzene	—	100	74	30	704	1	1
(4) Sample B, 0·3% in mouse fat ..	—	20	12	5	695	0	0
(5) Sample C, synthetic	—	20	11	6	5 alive day 440*	0	0
1:2-Diphenylchrysene	Cook and Galley (1931) ..	5	4	1	525	0	1
Fluoranthene	—	10	3	3	501	0	0
„	—	10	4	1	379	0	0
Chrysofluorene	Cook and Hewett (1934) ..	10	9	0	350	0	0
Benzanthrene	Clar and Furnari (1932) ..	20	14	9	608	0	0
1:9-Benzanthrone	—	10	7	0	294	0	0
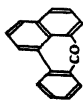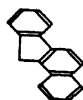
* On November 1, 1934.

† This compound was originally regarded as 1-methylpyrene, but has now been shown to be 2-methylpyrene by comparison with a sample supplied by Dr. G. Kränzlein, and synthesized by him by a method which establishes its constitution (private communication). The system of numbering which we adopt is that suggested by the International Committee on Organic Nomenclature, and differs from that used in continental publications.
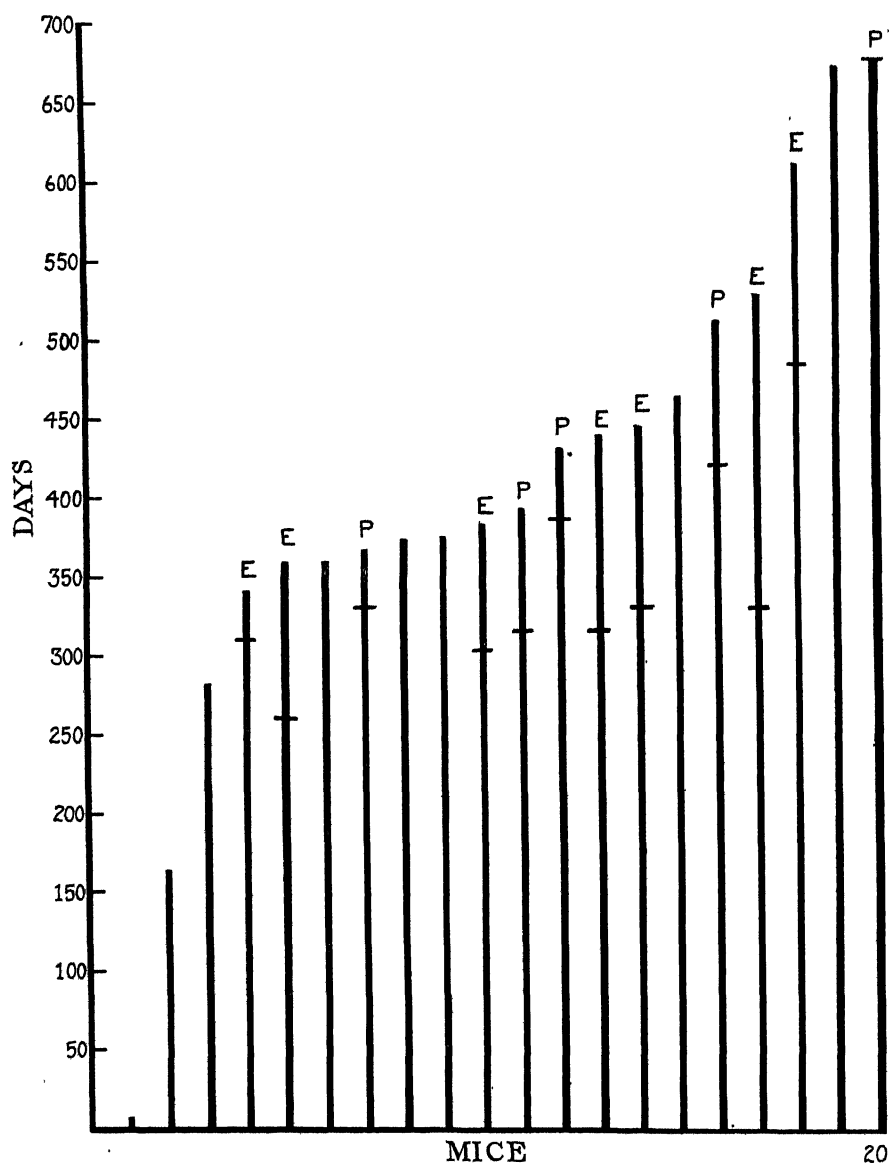
FIG. 1—3 : 4-Benzphenanthrene. 0·3% in benzene. (In this and figs. 2 to 7, each vertical line represents the duration of the life of one mouse after the commencement of painting. The transverse mark on some of the lines denotes the day when a tumour was first noticed. The letters at the tops of the lines show the results of microscopical examination of the tumours. E = epithelioma, P = papilloma. K. indicates that the mouse was killed to terminate the experiment.)

chrysene has any carcinogenic activity must remain in abeyance until the conclusion of experiments now in progress with synthetic chrysene. In our opinion, the results obtained with chrysene by Bottomley and Twort (1934), who claimed to have produced a considerable number of skin tumours in an unspecified number of mice, would have been more convincing had they been obtained with pure synthetic chrysene.

With pyrene also, occasional papillomas (3 in 45 mice) have been obtained; we are not prepared to assert that these were definitely due to the pyrene itself, as our earlier samples of this hydrocarbon which were used in these experiments were tinted yellow with impurities which we had been unable to remove. No tests have yet been completed with our later samples of pure, colourless pyrene. Table I shows also the negative

results obtained with fluoranthene, a somewhat analogous

hydrocarbon which is present in coal tar.

DERIVATIVES OF 1 : 2-BENZANTHRACENE

Tests have now been completed with a considerable number of simple homologues of 1 : 2-benzanthracene. Positive results are summarized in Table III and negative results in Table IV. From these results it is abundantly clear that the ring system of 1 : 2-benzanthracene gives a potentially carcinogenic molecule and that cancer-producing properties are developed by substitution in suitable positions. The activity of the 6-*iso*propyl compound in contrast with the negative results obtained with the 3-, 7-, and 10-*iso*propyl derivatives of 1 : 2-benzanthracene, the frequency with which tumours have arisen with other derivatives bearing substituents at positions 5 or 6, and the almost entire absence of tumours with numerous other methyl (3-, 7-, 2'-, 3'-) and dimethyl (2' : 6-, 2' : 7-, 3' : 6-, 3' : 7-) derivatives, all lend strong support to the view that attachment of alkyl groups to positions 5 or 6 (or both) of the 1 : 2-benzanthracene molecule gives molecular conditions which are favourable for cancer-producing activity.

All simple derivatives of 1 : 2-benzanthracene which have no substituent in positions 5 or 6 have so far given negative results, with the exception of the 4-methyl derivative, which gave one papilloma. It should be remarked, however, that no compounds with substituents at position 8 have yet been examined. Experiments described below with 1 : 2 : 7 : 8-dibenzanthracene and 3 : 4 : 5 : 6-dibenzacridine (pp. 327, 334) suggest that simple 8-alkyl derivatives of benzanthracene would be worthy of attention.

Although 6:7-*cyclo*penteno-1:2-benzanthracene is not strictly a homologue of benzanthracene, it may be classified among these for comparative purposes. The production of tumours with this compound has been definitely slower than with the isomeric 5:6-*cyclo*penteno-1:2-benzanthracene (Cook, 1932, *a*). This is exemplified in Table II.

TABLE II—TIME OF APPEARANCE OF TUMOURS OF SKIN

Produced by

5:6-*cyclo*Penteno-1:2-benzanthracene (50 mice)			6:7-*cyclo*Penteno-1:2-benzanthracene (10 mice)		
Day	Number of tumours	%	Day	Number of tumours	%
143 to 184 (41 days) ..	12	50·0	—	—	—
185 to 285 (100 days)..	10	41·7	240 to 285 (45 days)	2	28·6
286 to 305 (19 days) ..	2	8·3	286 to 353 (67 days)	5	71·4
	24	100·0		7	100
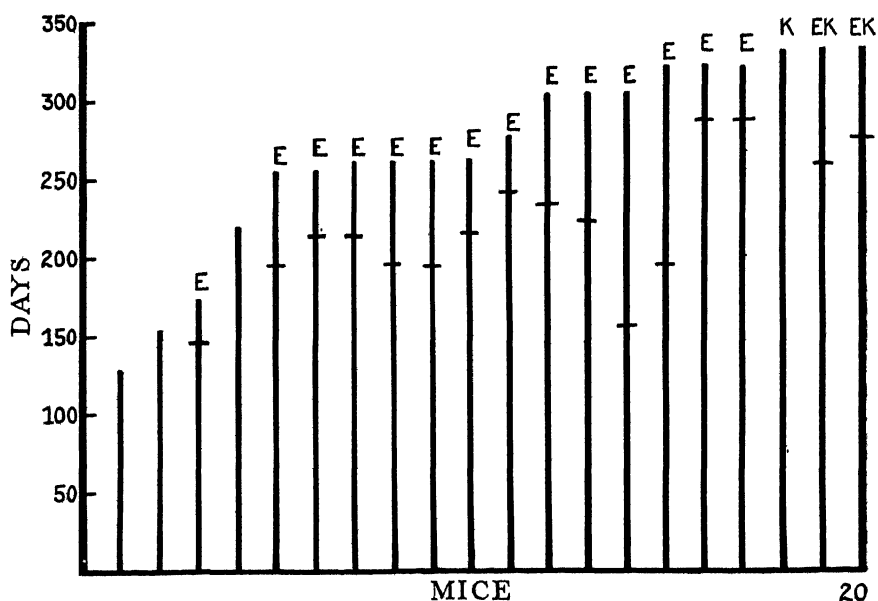
FIG. 2—5:6-Dimethyl-1:2-benzanthracene. 0·3% in benzene

Comparisons of 5-methyl-1:2-benzanthracene with the 6-methyl compound, of the 5:6-dimethyl homologue, fig. 2, with the 6:7-dimethyl compound, and of 5:6-*cyclo*penteno-1:2-benzanthracene with 6:7-*cyclo*penteno-1:2-benzanthracene point to the indubitable conclusion

TABLE III—1:2-BENZANTHRACENE AND ITS TUMOUR-PRODUCING HOMOLOGUES

Compound	Method of preparation	Mice				Tumours	
		Initial number	Alive after 6 months	Alive after 1 year	Day of death of last mouse	Epithelioma	Papilloma
1:2-Benzanthracene—							
(1) Not specially purified (2% in benzene)	Barnett and Matthews (1925)	50	13	2	574	0	0
(2) Purified through the picrate		30	10	7	584	1	0
4-Methyl-1:2-benzanthracene	Cook (1933)	10	3	0	312	0	1
5-Methyl-1:2-benzanthracene	,,	10	8	6	433	5	2
6-Methyl-1:2-benzanthracene	Cook (1932, b)	10	8	5	717	2	1
5:6-Dimethyl-1:2-benzanthracene	Cook and Haslewood (1934)	20	18	—	331	16	0
6:7-Dimethyl-1:2-benzanthracene—							
(1) Crude sample, m.p. 166–168°	Cook (1932, b)	10	10	5	588	3	1
(2) Pure sample, m.p. 173–174°		20	16	5	486	0	2
6-isoPropyl-1:2-benzanthracene—							
(1) Pure, m.p. 131–132°	,,	10	8	0	348	5	1
(2) Purified through quinone, m.p. 132–133°	,,	7	6	0	275	5	0
5:6-cycloPenteno-1:2-benzanthracene—							
(1) 0·3% in benzene	Cook (1931, d)	40	20	0	358	14	5
(2) 0·1% in benzene		10	6	0	339	5	0
(3) 0·03% in benzene		20	18	14	795	1	1
6:7-cycloPenteno-1:2-benzanthracene	,,	10	9	5	559	6	1
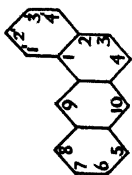
TABLE IV—NON-CARCINOGENIC SIMPLE DERIVATIVES OF 1:2-BENZANTHRACENE

Compound	Method of preparation	Mice				Tumours	
		Initial number	Alive after		Day of death of last mouse	Epithelioma	Papilloma
			6 months	1 year			
Dodecahydro-1:2-benzanthracene....	Cook and Hewett (1934)...	10	9		7 alive day 351*	0	0
3-Methyl-1:2-benzanthracene	Cook (1930)	14	9	4	458	0	0
7-Methyl-1:2-benzanthracene	Cook (1932, b)	10	5	4	563	0	0
2'-Methyl-1:2-benzanthracene	"	10	6	4	666	0	0
3'-Methyl-1:2-benzanthracene	"	10	3	3	924	0	0
2':6-Dimethyl-1:2-benzanthracene	"	10	7	6	674	0	0
2':7-Dimethyl-1:2-benzanthracene	"	10	8	6	786	0	0
3':6-Dimethyl-1:2-benzanthracene	"	10	9	6	707	0	0
3':7-Dimethyl-1:2-benzanthracene	"	10	9	8	612	0	0
3-isoPropyl-1:2-benzanthracene	"	20	9	7	554	0	0
7-isoPropyl-1:2-benzanthracene	"	10	9	6	794	0	0
10-isoPropyl-1:2-benzanthracene	"	10	6	3	611	0	0
6-Phenyl-1:2-benzanthracene	Cook (1930)	20	12	6	651	0	0
9:10-Diphenyl-1:2-benzanthracene	"	20	11	7	652	0	0
10-benzyl-1:2-benzanthracene (1·0, 0·5% in benzene)	"	10	8	3	466	0	0
Dimeride of 7-isoPropenyl-1:2-benzanthracene	Cook and Hewett (1933, b)	10	10	4	709	0	0

* On November 1, 1934.

that a substituent at position 5 is more efficacious in stimulating carcinogenic action than is the same substituent at position 6. The effect of introducing a single methyl group into a suitable position of the benzanthracene molecule is strikingly shown in the 5-methyl compound which has given 7 tumours (5 epitheliomas and 2 papillomas) in a series of 10 mice. It is also noteworthy, in connection with the feeble but definite activity of 6-methyl-1 : 2-benzanthracene, that both the 2′ : 6- and 3′ : 6-dimethyl derivatives of 1 : 2-benzanthracene have given negative results. This is in harmony with the observations previously recorded in the 1 : 2 : 5 : 6-dibenzanthracene series, that a methyl group at positions 2′ or 3′ has a depressant effect on the carcinogenic activity (Parts I and II).

<p style="text-align:center">PENTACYCLIC COMPOUNDS</p>

Of the 15 possible hydrocarbons containing systems of five condensed benzene rings in the molecule (formulæ shown below), 12 have now been tested for carcinogenic action, and of these only 2 have shown any pronounced activity. These are 1 : 2 : 5 : 6-dibenzanthracene and 1 : 2-benzpyrene. Tumour production by 1 : 2 : 5 : 6-dibenzanthracene has been described in previous communications, and the results with 1 : 2-benzpyrene are discussed below. In experiments carried out here 1 : 2 : 5 : 6-dibenzanthracene, when applied to the skin in eight different solvents, has given 126 epitheliomas and 41 papillomas in 413 mice. The three compounds (1 : 2 : 3 : 4-dibenzphenanthrene, 1 : 2 : 5 : 6-dibenzphenanthrene, 1′ : 2′-naphtha-1 : 2-anthracene) not yet tested are still unknown. Of the remaining 10 hydrocarbons, suggestions of very faint carcinogenic activity have been given in certain cases. Thus, 1 : 2 : 7 : 8-dibenzanthracene has given 1 epithelioma and 3 papillomas in 20 mice, in an experiment extending over 2 years, Table V. The tumours were very slow in making their appearance. Somewhat analogous results with 1 : 2 : 3 : 4-dibenzanthracene were recorded in Part I; as was pointed out, the material used in those experiments was not quite pure, and a further test in which pure 1 : 2 : 3 : 4-dibenzanthracene was applied to 20 mice has given no tumours. In this series the last mouse died on the 487th day, so that we cannot say that no tumours would have been obtained had the mice lived exceptionally long, as in the earlier series with the impure material (Part I, p. 466). 3 : 4 : 5 : 6-Dibenzphenanthrene gave only 2 transient papillomas. One epithelioma was also obtained in a series of 10 mice to which was applied a specimen of 4 : 5-benzpyrene isolated from coal tar (Table VII). We are reluctant to attribute this tumour, which first appeared after 13 months, to 4 : 5-benzpyrene, as the
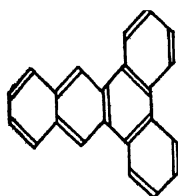
1 : 2 : 3 : 4-Dibenz-
anthracene
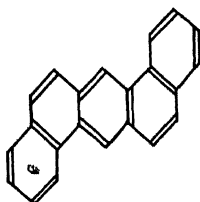
1 : 2 : 5 : 6-Dibenz-
anthracene
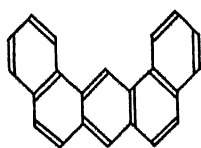
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anthracene
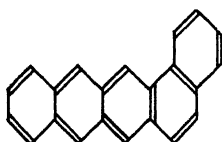
2′ : 3′-Naphtha-
2 : 3-phenanthrene
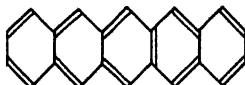
2 : 3 : 6 : 7-Dibenz-
anthracene
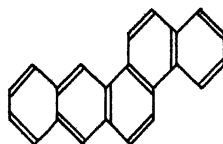
2′ : 1′-Naphtha-
1 : 2-anthracene
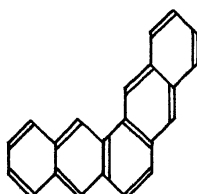
2′ : 3′-Naphtha-
1 : 2-anthracene
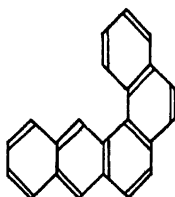
1′ : 2′-Naphtha-*
1 : 2-anthracene
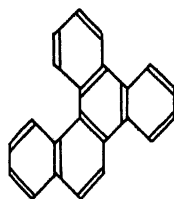
1 : 2 : 3 : 4-Dibenz-*
phenanthrene
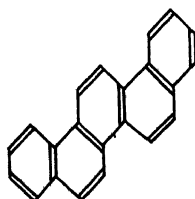
Picene
[1 : 2 : 7 : 8-Dibenz-
phenanthrene]
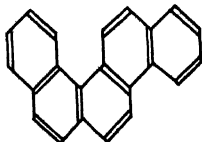
1 : 2 : 5 : 6-Dibenz-*
phenanthrene
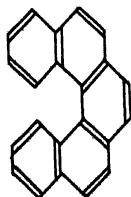
3 : 4 : 5 : 6-Dibenz-
phenanthrene
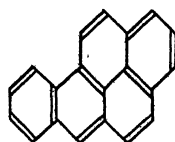
1 : 2-Benzpyrene
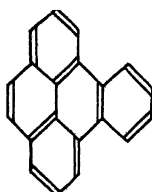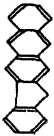
4 : 5-Benzpyrene
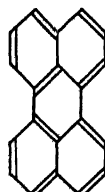
Perylene

* These three compounds are still unknown.

TABLE V—PENTACYCLIC AND HEXACYCLIC HYDROCARBONS

Compound	Method of preparation	Mice				Tumours	
		Initial number	Alive after 6 months	Alive after 1 year	Day of death of last mouse	Epithelioma	Papilloma
1:2:7:8-Dibenzanthracene	Cook (1932, c)	10	10	5	627	1	1
,,	Clar (1929)	10	9	7	722	0	2
2:3:6:7-Dibenzanthracene	Clar and John (1930)	20	11	2	487	0	0
,,		10	6	4	553	0	0
3:4:5:6-Dibenzphenanthrene	Cook (1933)	4.	2	0	313	0	0
2':3'-Naphtha-2:3-fluorene	Barnett, Goodway and Watson (1933)	10	7	6	613	0	0
2':3'-Phenanthra-1:2-anthracene (0·16% in benzene)	Cook (1931, b)	10	2	0	231	0	0
2':3'-Phenanthra-2:3-phenanthrene (0·25% in benzene)		10	7	4	754	0	0
Do. do. (0·17% in xylene)	,,	5	4	2	517	0	0
	,,	5	5	3	787	0	0

specimen used was certainly contaminated with other substances and a carcinogenic impurity, possibly 1:2-benzpyrene, may well have been present. No tests have been carried out with pure synthetic 4:5-benzpyrene.

COMPOUNDS RELATED TO 1:2:5:6-DIBENZANTHRACENE

The tests on some 20 simple derivatives of 1:2:5:6-dibenzanthracene are now almost complete, Table VI and Parts I and II, and the results fully justify the preliminary conclusion (Cook, 1932, *a*) that substitution, particularly in the *meso* positions, tends to diminish the carcinogenic potency of the 1:2:5:6-dibenzanthracene molecule. Reference to Table VI shows that, of the 20 mice to which the pure sample of phenanthra-acenapthene was applied, 7 developed tumours (4 epitheliomas and 3 papillomas). This is a simple derivative of 1:2:5:6-dibenzanthracene in which the *meso* positions are unsubstituted, and somewhat similar results were obtained with the 4-methyl, Table VI, and 2'-methyl, and 3'-methyl (*see* Parts I and II) derivatives of 1:2:5:6-dibenzanthracene. No compounds have been examined in which a single alkyl group is attached to a *meso* position, and it is impossible to assess the influence of the extraneous atoms introduced in the other 9-mono-substituted derivatives which have been examined (for example, the 9-methoxy and 9-amino compounds, which both gave positive results, and the 9-acetoxy compound, which gave negative results). It is perhaps not without significance, however, that the 9:10-dialkyl derivatives which have been examined, dimethyl, di-*n*-butyl, dibenzyl, Table VI and Part II, have given a comparatively small yield of tumours. For example, in an experiment which lasted more than 2 years, 9:10-di-*n*-butyl-1:2:5:6-dibenzanthracene gave only one epithelioma in a series of 10 mice. Similar loss of activity was observed with 9:10-dihydro-1:2:5:6-dibenzanthracene, which has given 2 tumours in a series of 10 mice; entirely negative results were obtained with *cis*-9:10-dimethyl-9:10-dihydro-1:2:5:6-dibenzanthracene (Part II). Thus any disturbance of molecular structure which affects the *meso* positions of 1:2:5:6-dibenzanthracene (either replacement of hydrogen, or reduction) seems to be attended by marked diminution of carcinogenic power. A comparison of 1:2:5:6-dibenzanthracene with its 9:10-dialkyl derivatives suggests that the property of carcinogenesis is facilitated (although not determined) by the presence of unsubstituted hydrogen atoms in the *meso* positions. These hydrogen atoms are readily removed by oxidation. It is certainly true that the active carcinogenic compounds are all extremely susceptible to the action of oxidizing agents, and the

role of these carcinogenic substances in producing tumours may be associated with some sort of interference with the normal oxidation processes of cells, occasioned by this ready oxidizability of the cancer-producing agents. That this cannot be the complete explanation of the cancer-producing action of the compounds is clear, for then the property of cancer-production would be much more common among many classes of easily oxidized substances. Attempts have been made to find some measure of explanation of carcinogenesis by hydrocarbons on lines such as these (Boyland, 1933; Pourbaix, 1933), but so far these attempts have not led to any conclusive results.

Most of the compounds tested in this Institute for carcinogenic action have been complex hydrocarbons and hence practically insoluble in water. Preliminary attempts have been made to find water-soluble carcinogenic agents by introducing into the 1:2:5:6-dibenzanthracene molecule groups which confer solubility in water. The biological effects of such compounds, administered intravenously, for example, would doubtless be of considerable interest. Inseparable mixtures of sodium salts of disulphonic acids of 1:2:5:6-dibenzanthracene were applied to the skin of mice and gave no tumours. Negative results were also obtained with a mixture of the sodium salts of 1:2:5:6-dibenzanthracene 4:8-dicarboxylic acid and 3:4:5:6-dibenzphenanthrene-1:8-dicarboxylic acid. More successful results were obtained with the sodium salt of 1:2:5:6-dibenzanthracene-9:10-endo-αβ-succinic acid (Cook, 1931, *e*)
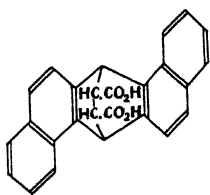
Subcutaneous injection of an aqueous solution of

this salt has given 6 tumours, mostly spindle-celled, in 60 mice. One of the tumours thus initiated has now reached the thirtieth transplanted generation, and is accompanied by leukæmia. These biological experiments were carried out by Mr. Burrows and extended by Mrs. Parsons, and will be described in detail in a subsequent communication.

In connection with the factor of oxidizability at the *meso* positions, it seemed of interest to examine compounds related to 1:2:5:6-dibenzanthracene in which one or both of the *meso* carbon atoms are replaced by nitrogen. Such compounds are 1:2:5:6-dibenzacridine and 1:2:5:6-dibenzphenazine.

1:2:5:6-Dibenzacridine proved, in fact, to be a cancer-producing compound and in the course of experiments extending over 482 days it

TABLE VI—DERIVATIVES AND HETEROCYCLIC ANALOGUES OF 1:2:5:6-DIBENZANTHRACENE

Compound	Method of preparation	Mice				Tumours	
		Initial number	Alive after 6 months	Alive after 1 year	Day of death of last mouse	Epithelioma	Papilloma
4-Methyl-1:2:5:6-dibenzanthracene	Cook (1933)	10	9	0	291	4	1
9:10-Di-n-butyl-1:2:5:6-dibenz-anthracene	Cook (1931, a)	10	8	1	742	1	0
9:10-Dihydro-1:2:5:6-dibenz-anthracene	Cook (1933)	10	7	1	426	2	0
9-Acetoxy-1:2:5:6-dibenzanthracene	Cook (1933)	10	9	5	678	0	0
9-Methoxy-1:2:5:6-dibenzanthracene	Cook (1931, e)	10	3	0	298	1	0
1:2:5:6-Dibenz-9-anthranol (not pure)	„	20	17	2	441	6	5
1:2:5:6-Dibenzanthraquinone	„	10	5	4	623	0	0
1:2:5:6-Dibenzanthraquinone	Clar (1929)	10	8	7	786	0	0
9-Nitro-1:2:5:6-dibenzanthracene	Cook (1931, e)	10	6	6	570	0	0
9-Amino-1:2:5:6-dibenzanthracene	„	10	4	4	538	0	0
9-n-Butyrylamino-1:2:5:6-dibenzanthracene	„	10	7	5	632	3	1
N-1:2:5:6-Dibenzanthranyl-succinimide	„	10	3	2	476	0	0
1:2:5:6-Dibenzanthracene-9:10-endo-$\alpha\beta$-succinic acid	„	10	5	2	569	0	0
	„	10	8	8	724	·	~

Substance	Reference						
Phenanthra-acenaphthene (pure)	Cook (1931, b)	5	3	2	577	2	0
"		5	3	2	677	0	1
"		10	9	6	539	2	2
1:2:5:6-Dibenzacridine	Ullmann and Fetvadjian (1903)	10	7	0	349	1	0
3:4:5:6-Dibenzacridine	Möhlau and Haase (1902)	30	27	12	482	4	2
"		10	6	3	597	2	0
1:2:5:6-Dibenzphenazine	Reitzenstein and Andre (1913)	30	28	18	551	9	2
"		20	16	8	660	0	0
Isonaphthathioxin	Cohen and Smiles (1929)	10	9	—	5 alive day 295*	—	—

* On November 1, 1934.

yielded 7 tumours (5 epitheliomas and 2 papillomas) in 40 mice, fig. 3. Of considerable interest, also, is the fact that an allied substance, 3 : 4 : 5 : 6-dibenzacridine was also carcinogenic, fig. 4. Indeed, it seemed more active than the 1 : 2 : 5 : 6-compound, for 13 tumours (11 epitheliomas and 2 papillomas) were obtained in two series com-
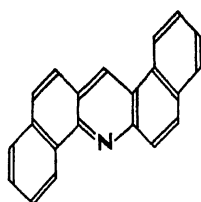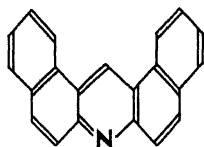
1 : 2 : 5 : 6-Dibenzacridine   3 : 4 : 5 : 6-Dibenzacridine   1 : 2 : 5 : 6-Dibenzphenazine
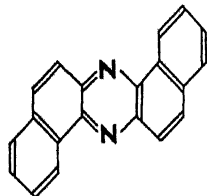
prising 40 mice in all. The action of these two compounds is slow; only 5 of the 17 tumours represented in figs. 3 and 4 appeared before the 300th day.

This 3 : 4 : 5 : 6-dibenzacridine bears the same relationship to 1 : 2 : 5 : 6-dibenzacridine as 1 : 2 : 7 : 8-dibenzanthracene does to 1 : 2 : 5 : 6-di-benzanthracene (cf. formulæ on p. 328), at least so far as molecular
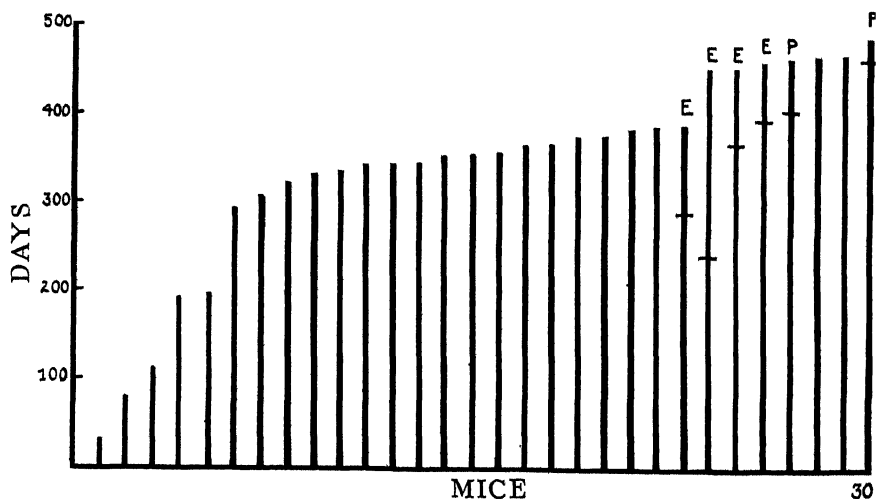
Fig. 3—1 : 2 : 5 : 6-Dibenzacridine.   0·3% in benzene.

structures are concerned, and it will be recalled that 1 : 2 : 7 : 8-dibenz-anthracene had very little cancer-producing activity (p. 327). Relation-ships such as these indicate the difficulties in arriving at any complete generalization regarding the molecular conditions which are necessary for cancer-producing activity. The parent compound acridine has been

tested on the skin of 200 mice with negative result (Kennaway, 1924). Proflavine (diaminoacridine sulphate) and acriflavine (diamino-methyl-acridinium hydrochloride) have given negative results in painting experiments of 488 days' duration.

1 : 2 : 5 : 6-Dibenzphenazine was also examined for comparison with the corresponding anthracene and acridine derivatives. It has been applied to the skin of mice in the usual way, but so far with completely negative results. In this connection, one may point out that in this di-benzphenazine there are no longer the possibilities of ready oxidation at
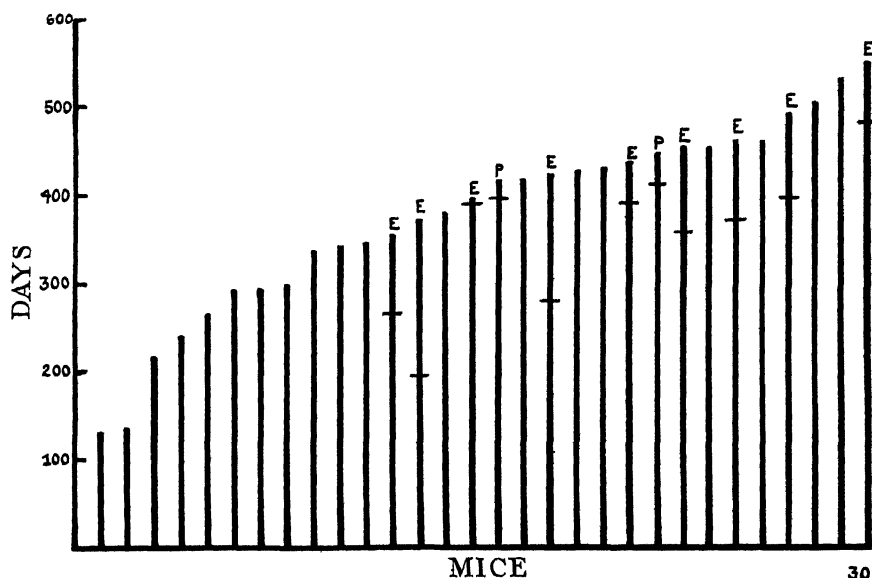
FIG. 4—3 : 4 : 5 : 6-Dibenzacridine. 0·3% in benzene.

the *meso* positions as is the case with 1 : 2 : 5 : 6-dibenzanthracene and the dibenzacridines.

A sulphur compound related to 1 : 2 : 5 : 6-dibenzacridine, namely, *iso*naphthathioxin has so far given no tumours in a series of 10 mice of which 5 are still alive after 295 days.

1 : 2-BENZPYRENE

This hydrocarbon is the only one of the carcinogenic substances so far examined which has been shown to be present in coal tar. It was first isolated from coal tar pitch and was afterwards prepared synthetically (Cook, Hewett and Hieger, 1933) from pyrene by the sequence of changes shown below.

It will be seen from its formula that 1 : 2-benzpyrene contains the ring systems of pyrene, chrysene and 1 : 2-benzanthracene. The actual " fine structure " of the molecule is probably more closely related to 1 : 2-benzanthracene than to the other two hydrocarbons, for the fluorescence spectrum is of exactly the same type as that of 1 : 2-benzanthracene, but is appreciably different from the spectra given by chrysene and pyrene (Hieger, 1930). Comparison of the absorption spectrum of 1 : 2-benz-pyrene with those of the three parent hydrocarbons under discussion

Pyrene
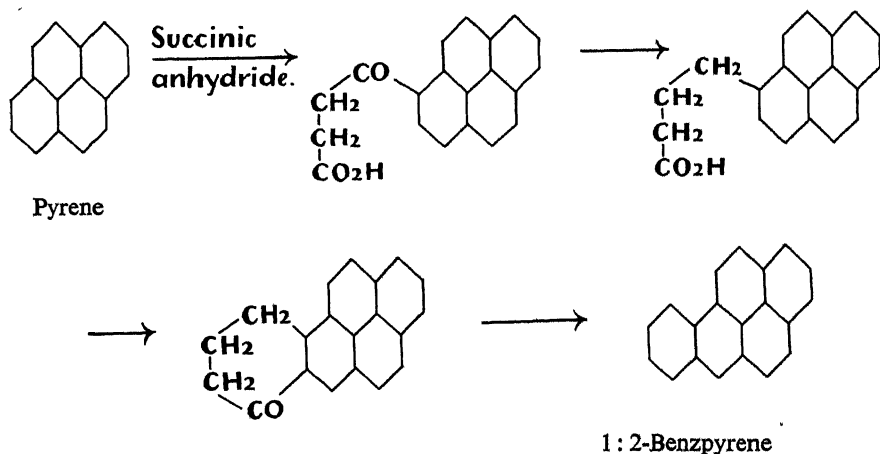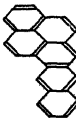
1 : 2-Benzpyrene

shows also that the benzpyrene spectrum is definitely of the benzanthracene type, although some of the features of the pyrene spectrum may also be detected. These spectroscopic comparisons have been made by Dr. W. V. Mayneord and Miss Roe, B.Sc., and will be published subsequently.

The results obtained by the application of 1 : 2-benzpyrene to mice, figs. 9 and 10, Plate 20, are summarized in Table VII, which shows that the material isolated from pitch gave results quite parallel with those obtained with the synthetic sample. In all, 58 tumours (47 epitheliomas and 11 papillomas) have been obtained in 100 mice, and from this high incidence as also from the early appearance of tumours, figs. 5 and 6, it is clear that 1 : 2-benzpyrene is a considerably more potent carcinogenic agent than 1 : 2 : 5 : 6-dibenzanthracene, or, in fact, than any compound already discussed. In recent publications, Maisin and Liégeois (1934) and Maisin and De Jonghe (1934) stated that they had examined a sample of 1 : 2-benzpyrene prepared synthetically by the method of Cook Hewett and Hieger (1933) and that they had found it to be much more active in producing tumours than the material which we used. Since

TABLE VII—1:2-BENZPYRENE AND RELATED COMPOUNDS

Compound	Method of preparation	Initial number	Alive after		Day of death of last mouse	Tumours	
			6 months	1 year		Epi-thelioma	Papil-loma
1:2-Benzpyrene—	Cook, Hewett and Hieger (1933)						
A. Synthetic—							
Series 1		10	3	0	206	4	1
Series 2		10	4	1	368	3	0
Series 3		10	2	0	221	5	2
*Series 4. 1:2-Benzpyrene picrate		10	6	0	353	5	0
*Series 5. Mixture of 1:2-benzpyrene (3 parts) and 1:2-benzpyrene picrate (1 part)		10	8	1	368	5	1
B. Isolated from coal-tar pitch—							
Sample 1, m.p. 177·5–178·5°		10	10	0	295	6	3
Sample 2, m.p. 175·5–176·5°		10	3	0	202	6	1
Sample 3, m.p. 175·5–176·5°		10	5	0	225	8	2
Sample 3, (0·03% in benzene)		10	10	9	638	3	0
„ „ „		10	9	3	503	2	1
4:5-Benzpyrene (isolated from coal tar pitch, not completely purified)	Cook, Hewett and Hieger (1933)	10	9	5	660	1	0
1-Phenyl-2:3:4:5-dibenzpyrene	Clar (1930)	10	10	3	635	0	1
2′-3′-Naphtha-1:2-pyrene	Cook, Hewett and Hieger (1933)	20	20	15	713	0	0

* Results show that addition of picric acid did not affect tumour production.

we have not hitherto published any details of our experiments, it is difficult
to understand the basis of this claim.   Actually, the data which we now
record are in fair agreement with those published by Maisin, Liégeois and
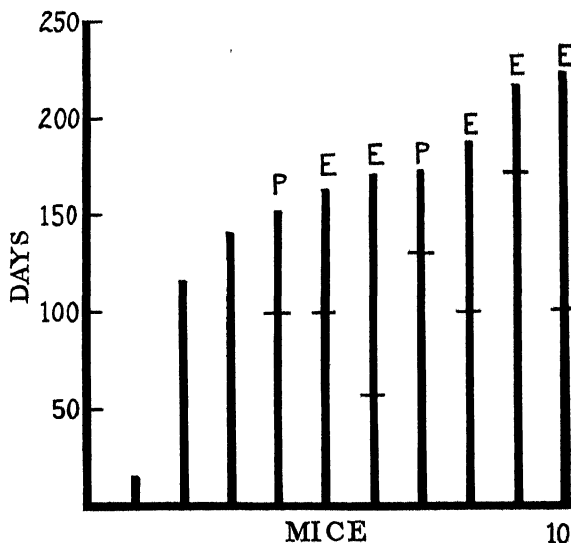
FIG. 5—Synthetic 1 : 2-benzpyrene.   0·3% in benzene
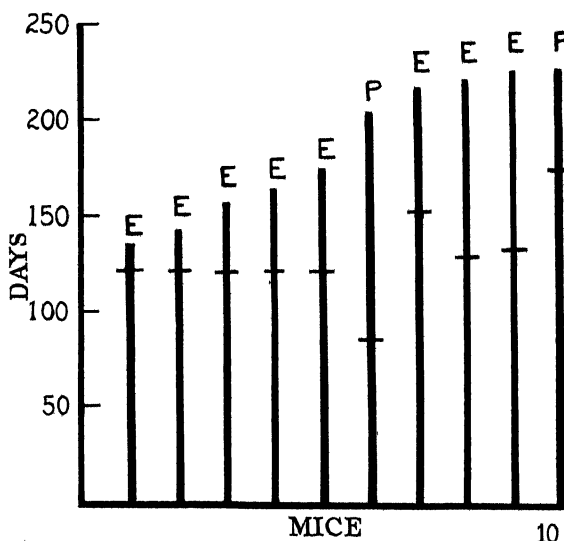
FIG. 6—1 : 2-Benzpyrene isolated from coal-tar pitch.   0·3% in benzene

De Jonghe.   The differences in physical properties between their 1 : 2-
benzpyrene and our own highly purified synthetic samples are clearly
due to the presence of a small amount of impurity in the material which

they used. Their suggestion that they were dealing with " une variété particulière de cette substance " is completely meaningless.

2′: 3′-Naphtha-1: 2-pyrene, in which an additional benzene ring is condensed with the 1: 2-benzpyrene molecule, has given no tumours in a series of 20 mice, of which 15 lived for more than one year, Table VII.

TUMOURS OF CONNECTIVE TISSUE PRODUCED BY 1: 2-BENZPYRENE

No subcutaneous injections of the purest samples of 1: 2-benzpyrene have been made. A rat which had received *sub cutem* 0·1 gm of moderately pure 1: 2-benzpyrene (m.p. 168–170°), from pitch, in 3 cc sesame oil in the course of tests by Professor E. C. Dodds for œstrogenic action (Cook, Dodds, Hewett and Lawson, 1934), developed a spindle-celled tumour at one of the sites of injection. An autograft made on the 165th day showed growth when the rat was killed on the 181st day. Grafts of the tumour died out in the second generation.

Two series of 10 mice each received *sub cutem*, usually at weekly intervals, crystalline fractions, which consisted very largely of 1: 2-benzpyrene, obtained in the course of isolation from pitch. Series A received a fraction of m.p. about 160°, series B one of m.p. 164–168° (both 0·3% in lard). Series A yielded 4, and series B 5, spindle-celled tumours, the 9 mice being killed between the 115th and 191st days (average 146th day); grafts made from one of these tumours did not persist beyond the second generation. These figures indicate a rapid growth of the tumour. Thus, in earlier experiments (Burrows, Hieger and Kennaway, 1932; Barry and Cook, 1934) 31 mice bearing primary tumours caused by 1: 2: 5: 6-dibenzanthracene were killed between the 144th and 243rd days (average 186th day) and 4 mice bearing primary tumours caused by 5: 6-*cyclo*penteno-1: 2-benzanthracene were killed between the 196th and 280th day (average 250th day).

Maisin and Coolen* (1934) have produced tumours by subcutaneous injection in rats of 1: 2-benzpyrene dissolved in lard. " Ces tumeurs ont une évolution extrêment rapide, 15 à 25 jours après l'apparition du 1ᵉʳ nodule les animaux meurent du développement énorme du néoplasme qui peut donner des métastases multiples (rein, poumon, pancréas, cœur, etc.). Ces tumeurs sont très anaplasiques et certaines aussi semblent bien être des myosarcomes. Elles naissent plus tôt qu'après injection de 1-2 5-6 dibenzène anthracène."

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* These authors credit us with experiments which we have never performed on the production of sarcomas in rats by 1: 2-benzpyrene.
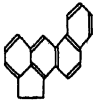
METHYLCHOLANTHRENE

The investigations relating to pure synthetic carcinogenic compounds of known molecular structure have assumed an entirely new aspect with the recognition and proof of the true molecular structures of the group of natural products which comprise the sterols and bile acids. For it is now clear that there is a definite similarity of molecular structure between these natural products and the cancer-producing hydrocarbons. In particular, the sterols and bile acids contain in their molecules condensed carbon-ring systems to which is attached a long side-chain in such a position that a new six-membered ring can be formed so as to give the 1 : 2-benzanthracene ring system without molecular rearrangement or group migration. The purely aromatic hydrocarbon, methyl-

Deoxycholic acid   12-Ketocholanic acid

Dehydronorcholene   Methylcholanthrene
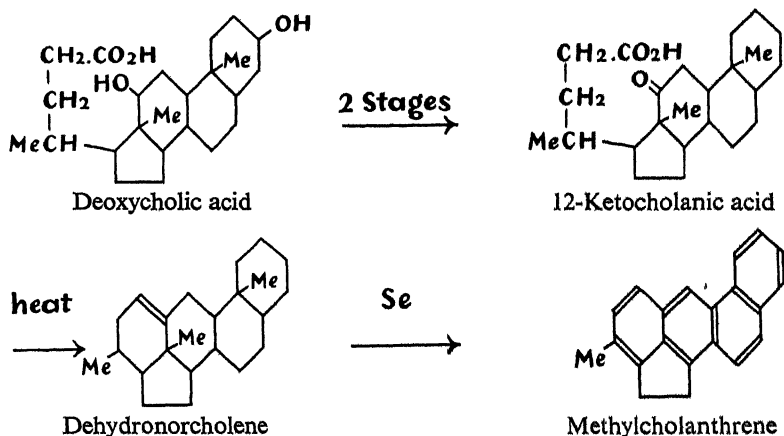
cholanthrene,* obtained thus from the bile acid, deoxycholic acid, by the above series of changes, is a benzanthracene hydrocarbon with substituents in positions 5 and 6. This is a favourable type of molecular structure for the development of carcinogenic properties (p. 323).

* Wieland and Dane (1933) have suggested the name " cholanthrene " for the hypothetical anthracenoid hydrocarbon from which such aromatic hydro-carbons related to the cholane group are derived.

[*Note added in proof*, March 19, 1935.—The synthetic preparation of methyl-cholanthrene has recently been announced by L. F. Fieser and A. M. Seligman (' J. Amer. Chem. Soc.,' vol. 57, p. 228, (1935)), and the parent hydrocarbon cholan-threne, m.p. 170°, has now been synthesized in this Institute by J. W. Cook, G. A. D. Haslewood, and Mrs. A. M. Robinson.]

The structure of methylcholanthrene has been established by the synthesis of a degradation product (5: 6-dimethyl-1: 2-benzanthraquinone) (Cook and Haslewood, 1934). Moreover, methylcholanthrene has proved to be a potent cancer-producing hydrocarbon, the order of activity of which is at least equal to that of 1: 2-benzpyrene, and possibly slightly greater. The high activity of methylcholanthrene is shown not only by the early appearance of tumours, but also in the very high proportion of animals which develop tumours, fig. 7 and Table VIII. In one series of 20 mice, 18 tumours (17 epitheliomas and one papilloma) were obtained in an experiment which was ended in 180 days, and in another series the first tumour appeared after 31 days. Fig. 11, Plate 20, illustrates a metastasis obtained in another series of mice, in which a dilute solution of very highly purified methylcholanthrene was employed. Methylcholanthrene thus establishes a clear connecting link between the carcinogenic hydrocarbons and the sterols and bile acids, and it is of great interest that the changes by which it is obtained from deoxycholic acid are all reactions of the type which are known to occur normally in the animal body, although there is no evidence that this particular sequence of changes involved in the formation of methylcholanthrene does actually occur in nature.

TUMOURS OF CONNECTIVE TISSUE PRODUCED BY METHYCHOLANTHRENE

Four earlier papers from this Institute have described spindle-celled tumours of connective tissue produced by 1: 2: 5: 6-dibenzanthracene (Burrows, Hieger and Kennaway, 1932; Burrows, 1932 and 1933; Barry and Cook, 1934), and by 5: 6-*cyclo*penteno-1: 2-benzanthracene and chrysene (Barry and Cook, 1934). Methylcholanthrene was dissolved in lard liquid at 37° (2 or 3 mg in 1 cc) by heating in boiling water, and injections of 1 cc of this solution into the right groin of 15 rats were made at intervals, usually of 1 week. Tumours arose, fig. 8, in each one of the 9 rats out of the original 15 which lived for more than 70 days, the tumour-bearing animals being killed between the 123rd and 193rd day (average 162nd day); the comparable average figures obtained here for duration of life in experiments on rats with other hydrocarbons are:—

	Days
1: 2: 5: 6-dibenzanthracene (2 series)	210, 193
, 5: 6-*cyclo*penteno-1: 2-benzanthracene	422
Chrysene...........................	449

Hence methylcholanthrene produces tumours of this type more rapidly than does any other hydrocarbon which we have tested as yet.

The structure of these tumours is spindle-celled, figs. 12–15, Plate 21, and they have on the whole a more fibromatous appearance than those
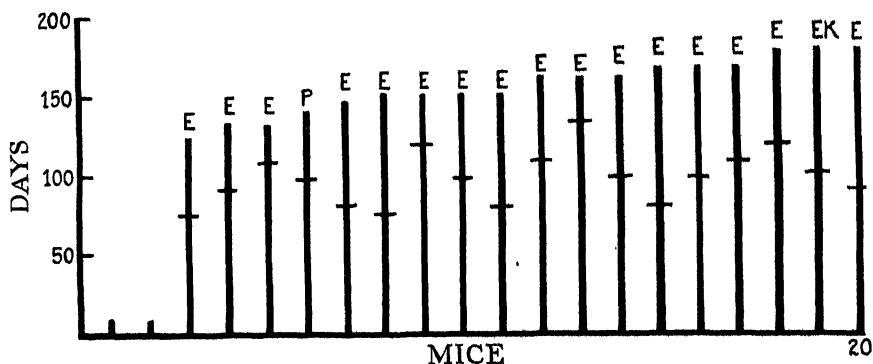
Fig. 7—Methylcholanthrene. 0·3% in benzene

produced by other hydrocarbons. Numerous giant cells, fig. 14, Plate 21, are present in some of them (cf. Burrows, 1932, fig. 9). The invasive powers of these tumours appear to be rather low in spite of their great size,
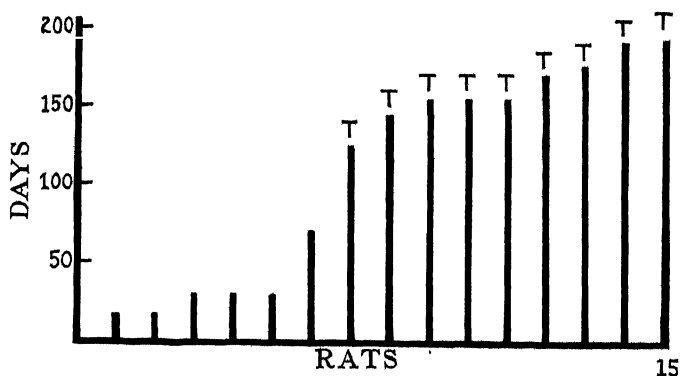
Fig. 8—Methylcholanthrene in lard, 2 or 3 mg in 1 cc. Connective tissue tumours in rats. Each vertical line represents the duration of the life of one rat from the beginning of the experiment. T = tumour present at death

but they have given more vigorous autografts than have any of the earlier series, fig. 13, Plate 21. The only metastases found have been single nodules—one of these 30 mm diameter—in the left flank of two rats. Four heterografts were carried out; three were negative and the fourth failed after the third generation (cf. a rat tumour, LR 10, produced in

TABLE VIII—METHYLCHOLANTHRENE

Compound	Mice				Tumours	
	Initial number	Alive after		Day of death of last mouse	Epi-thelioma	Papil-loma
		6 months	1 year			
Methylcholanthrene	20	0	0	179	17	1
Methylcholanthrene (0·06% in benzene)	20	12	0	268	7	4
Methylcholanthrene (very highly purified)	10	5	0	198	6	1
„ „	5	0	0	164	2	3
Methylcholanthrene (very highly purified, 0·06% in benzene)	10	6	0	252	5	1

this laboratory by 1 : 2 : 5 : 6-dibenzanthracene, which has now reached the 71st transplanted generation).*

It is noteworthy that the various attributes of a malignant mesodermal neoplasm (rapid growth; cellular structure; invasive power; formation of metastases; growth in autograft, and in heterograft) appear to be by no means uniformly distributed among the tumours produced by the five hydrocarbons which have been used (1 : 2 : 5 : 6-dibenzanthracene, 5 : 6-*cyclo*penteno-1 : 2-benzanthracene, chrysene, methylcholanthrene, 1 : 2-benzpyrene). Those due to methylcholanthrene show rapid growth and good development of autografts but are more or less deficient in the other characters.

The same solution of methylcholanthrene in lard was injected *sub cutem* in 10 mice; some ulceration occurred and there was a high death rate. Eight of the 10 mice died, showing no tumours, before the 148th day. The remaining 2, killed on the 152nd and 154th days, bore tumours at the site of injection which were spindle-celled and similar in microscopic appearance to those produced in mice by 1 : 2 : 5 : 6-di-benzanthracene and 5 : 6-*cyclo*penteno-1 : 2-benzanthracene. Grafts of these tumours gave negative results.†

STEROLS, ŒSTRINS AND RELATED COMPOUNDS

Certain sterols and their derivatives (ergosterol, calciferol, neo-ergosterol, cholestene) and keto-hydroxyœstrin (œstrone) and some derivatives and allied compounds (tri-hydroxyœstrin, keto-methoxy-œstrin, equilin, equilenin, pregnandiol) were tested for carcinogenic action by painting on the skin of mice, Table IX. These experiments have been entirely negative, but one must bear in mind that with œstrin and some of its derivatives the life of the animals, and hence the time of exposure to the compound in question, is shortened by various obstruc-tive effects upon the genito-urinary tract (pyometra, hydronephrosis, abscess of the coagulating gland) (Lacassagne, 1932; Burrows and Kennaway, 1934). We have not yet used solutions so dilute that these effects are absent, or of slight degree.

* The use of heterografts as a test for malignancy in tumours of this type requires to be considered in the light of the recent work of Andervont (1934), who finds that spindle-celled tumours produced by 1 : 2 : 5 : 6-dibenzanthracene in pure-strain mice will grow only in mice of the same strain.

†[*Note added in proof*, March 19, 1934.—Three more spindle-celled tumours have been obtained from 10 mice injected *sub cutem* with methylcholanthrene, the tumour-bearing animals being killed on the 116th, 116th and 120th days. One of these tumours is growing in the 3rd grafted generation.]

FIG. 9

FIG. 10
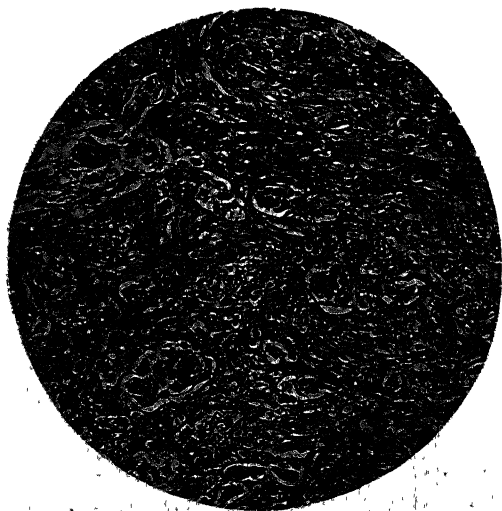
FIG. 11
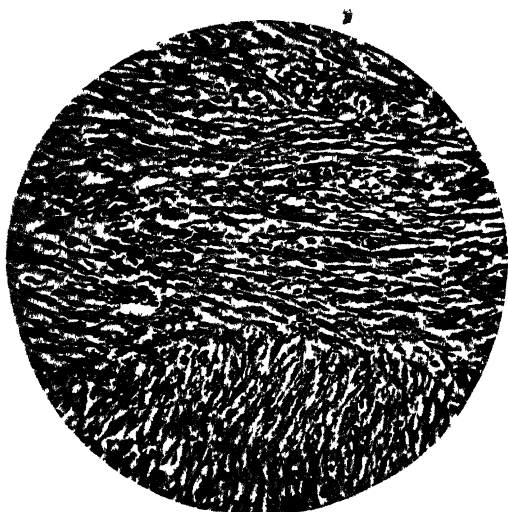
FIG 12

FIG 13
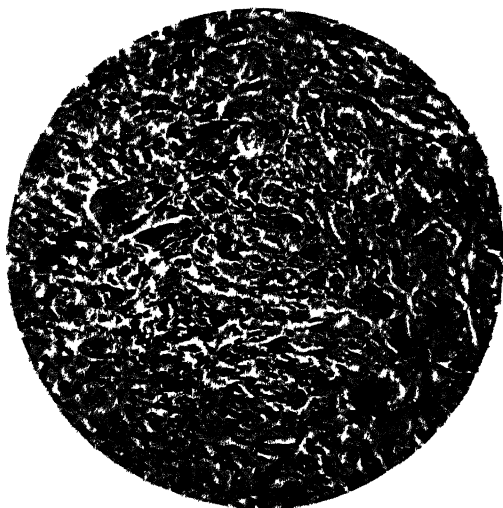
FIG 14
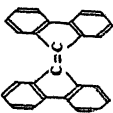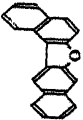
FIG 15

TABLE IX—STEROLS, ŒSTRINS AND RELATED COMPOUNDS

Compound	Method of preparation	Concentration % in benzene	Initial number	Mice Alive after 6 months	Mice Alive after 1 year	Day of death of last mouse	Duration of experiment on 1.11.34 (in days)	Tumours Epithelioma	Tumours Papilloma
Ergosterol		0·3	50	34	11	1 alive	597	0	0
,,		0·3	20♀	18	18	4 alive	583	0	0
Calciferol		0·3	50	29	11	518	—	0	0
,,		0·3	20♀	15	11	5 alive	583	0	0
Neoergosterol	Inhoffen (1932)	0·3	20	10	6	2 alive	511	0	0
Cholestene		0·3	20	14	7	587	—	0	0
Ketohydroxyœstrin— (1)		0·1	30	7	1	372	—	0	0
(2)		0·01	20♂	3	1	378	—	0	0
(3)		0·01	20♀	18	11	5 alive	422	0	0
(4)		0·01	11♂ C	10	6	6 alive	391	0	0
Ketomethoxyœstrin— (1)		0·1	20♀	15	—	6 alive	321	0	0
(2)		0·1	10♀ C	5	—	5 alive	282	0	0
(3)		0·1	20♂	12	0	5 alive	296	0	0
(4)		0·1	20♂	2		245	—	0	0
(5)		0·1	6♂ C	4	—	2 alive	258	0	0
Trihydroxyœstrin (0·1% in alcohol)		—	5	3	0	3 alive	236	0	0
Equilin		0·1	5♂	4	0	246	—	0	0
Equilenin		0·1	5♂	5	0	245	—	0	0
Pregnandiol (0·3% in alcohol)		—	20	16	9	8 alive	369	0	0
1:2-*cyclo*Pentenophenanthrene	Cook and Hewett (1933, *a*)	0·3	20	15	7	439	—	0	0
1:9-Dimethylphenanthrene	Haworth and Mavin (1932)	0·3	30	24	21	8 alive	468	0	0
,,		0·3	20	15	6	2 alive	490	0	0
1-Keto-1:2:3:4-tetrahydro-phenanthrene	Haworth (1932)	0·3	10♂	10	6	535	—	0	0
		0·3	10♀	9	9	5 alive	583	0	0

C = castrated.

TABLE X—OTHER COMPOUNDS

Compound	Method of preparation	Mice				Duration of experiment on 1.11.34 (in days)	Tumours	
		Initial number	Alive after		Day of death of last mouse		Epithelioma	Papilloma
			6 months	1 year				
Synthetic anthracene		50	34	16	5 alive	615	0	0
bis-Diphenylene ethylene ..		10	8	6	553	—	0	0
7:8-Dihydrophenalyl-7-*spirocyclopentane*	Cook and Hewett (1934)	20	15	4	3 alive	492	0	0
" "		10	10	10	5 alive	478	0	0
Carotene		10	7	1	488	—	0	0
βββα-Dinaphthafuran		10	4	2	486	—	0	?1

1 : 2-*cyclo*Pentenophenanthrene may be regarded as the fundamental aromatic hydrocarbon from which are derived not only the sterols and bile acids, but also the œstrus-producing hormones keto- and trihydroxy-œstrin, equilin, and equilenin.   It has been tested on the skin in series of 50 mice in all for now nearly 18 months, with negative results, and it has been injected *sub cutem* (2 mg in 1 cc lard), usually at weekly intervals, in 10 rats of which 8 are now alive (326th day) and show no tumours. This compound has shown no œstrogenic action (Cook, Dodds, Hewett and Lawson, 1934).   Thus various biological effects brought about by
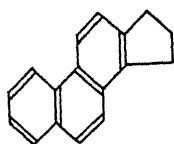
1 : 2-*cyclo*Penteno-
phenanthrene
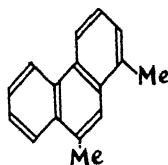
1 : 9-Dimethyl-
phenanthrene
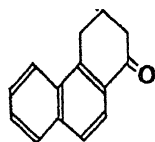
1-Keto-1 : 2 : 3 : 4-
tetrahydrophen-
anthrene

compounds which may be regarded as derived from 1 : 2-*cyclo*penteno-phenanthrene must depend upon modifications of the parent molecule. 1 : 9-Dimethylphenanthrene and 1-keto-1 : 2 : 3 : 4-tetrahydrophenanthrene have given no epithelial tumours.   The latter compound gave, on sub-cutaneous injection in 5 rats, 2 tumours of polymorphous cellular structure; these will be described by Mr. Burrows in another com-munication.

DISCUSSION

We have now examined for cancer-producing activity 71 compounds which are not related in molecular structure to 1 : 2-benzanthracene, and 65 of these have given entirely negative results.   Of the 6 compounds which gave positive results only 3 : 4-benzphenanthrene had considerable activity, fig. 1.   The remaining 5 comprised pyrene (p. 323) and chry-sene (p. 319) and their derivatives 4 : 5-benzpyrene, 1-phenyl-1 : 3 : 4 : 5-dibenzpyrene and 1 : 2-diphenyl chrsyene.   With the exception of chrysene (which gave 1 epithelioma and 3 papillomas in 290 mice) and an impure specimen of 4 : 5-benzpyrene (which produced 1 epithelioma in 10 mice, *see* p. 327), these compounds have given only occasional papillomas.   βββα-Dinaphthafuran gave one doubtful papilloma.

In all, 69 compounds related to 1 : 2-benzanthracene have now been tested and 25 of these have given positive results.   With very few

exceptions, the active compounds are all derivatives of 1 : 2-benzanthracene containing substituents at positions 5 or 6, or both, these substituents being (*a*) alkyl groups, as in 5-methyl- and 6-*iso*propyl-1 : 2-benzanthracene, (*b*) saturated rings, as in 5 : 6-*cyclo*penteno-1 : 2-benzanthracene and methylcholanthrene, or (*c*) an additional aromatic ring, *i.e.*, in 1 : 2 : 5 : 6-dibenzanthracene and its derivatives. Analogous to the latter group is 1 : 2 : 5 : 6-dibenzacridine. The active compounds related to 1 : 2-benzanthracene which do not come within these categories are 1 : 2-benzpyrene and 1 : 2: 7 : 8-dibenzanthracene, and its analogue 3 : 4 : 5 : 6-dibenzacridine. 1 : 2-Benzpyrene is more active than any other compound examined except methylcholanthrene, and forms an exception to the generalization that substituents in the 5 and 6 positions of the benzanthracene molecule are necessary for a high order of carcinogenic activity. However, 1 : 2-benzpyrene is not of the same type as the other benzanthracene derivatives under discussion, as it contains in its molecule a more highly condensed aromatic ring system than these.

The 44 compounds related to 1 : 2-benzanthracene which have given negative results are for the most part derivatives in which the substituents have been attached to the molecule at positions other than 5 and 6, or else compounds related to 1 : 2 : 5 : 6-dibenzanthracene by the attachment of additional benzene rings. The lack of activity of such hexacyclic, heptacyclic and octacyclic derivatives of 1 : 2 : 5 : 6-dibenzanthracene is in keeping with the view that there is an optimum state of molecular complexity for carcinogenic activity. In this connection it is noteworthy that an additional benzene ring destroys the activity of the 1 : 2-benzpyrene ring system, Table VII. Attention has been drawn (p. 330) to the suppression of carcinogenic activity brought about by reduction of 1 : 2 : 5 : 6-dibenzanthracene or by substitution in positions 9 and 10.

The particular compounds which we have tested for cancer-producing action are mostly polycyclic aromatic hydrocarbons or closely related compounds. Our attention was especially directed to this group of compounds in the course of investigations on the chemical nature of the carcinogenic constituents of coal tar and other carcinogenic mixtures of like nature. We fully recognize that there may be other classes of carcinogenic substances of quite different chemical type from those with which we have been concerned. In connection with such a possibility, we may observe that Browning, Cohen, Cooper, Ellingworth and Gulbransen (1933) have recorded the development of a sarcomatous growth following the injection into mice of 2-(*p*-aminostyryl)-6-(*p*-acetylamino-benzoylamino) quinoline methoacetate, a substance which they had prepared and tested for trypanocidal action.

Table XI summarizes the number of mice used and the number of tumours obtained in tests for carcinogenic activity carried out in this Institute with 140 compounds.

TABLE XI

Classes of compounds	Number of compounds tested	Number of mice	Tumours produced	
			Epithe-lioma	Papil-loma
Not related to 1 : 2-benzanthracene—				
Negative	65	2026	—	—
Positive	6	380	9	13
Related to 1 : 2-benzanthracene—				
Negative	44	619	—	—
Positive	25	1220	335	102
	140			

Thus, the 6 compounds not related to 1 : 2-benzanthracene which have given positive results gave 22 tumours (9 epitheliomas and 13 papillomas) in 380 mice. Twelve of these tumours (7 epitheliomas and 5 papillomas) were produced by 3 : 4-benzphenanthrene. On the other hand, the 25 carcinogenic compounds which are related to 1 : 2-benzanthracene have given 437 tumours (335 epitheliomas and 102 papillomas) in 1220 mice.

assistants, whose good work has in various ways helped these investigations.

SUMMARY

Tests for cancer-producing action have been carried out upon a large number of compounds, chiefly tetracyclic and pentacyclic aromatic hydrocarbons. Two derivatives of 1:2-benzanthracene which have considerable cancer-producing power are 1:2-benzpyrene, a compound isolated from coal tar pitch, and methylcholanthrene, a compound derived from the deoxycholic acid of bile. Substitution in the 5- and 6-positions of the 1:2-benzanthracene molecule is important in the development of carcinogenic properties. The only compound not related to 1:2-benzanthracene which has shown any considerable carcinogenic power is 3:4-benzphenanthrene.

DESCRIPTION OF PLATES

PLATE 20

FIGS. 9–11—Metastases from cancer of skin in mice after painting with pure hydrocarbons dissolved in benzene.

FIG. 9—1:2-Benzpyrene, 0·3%. Axillary gland. Day 199. × 26. 7/33.

FIG. 10—1:2-Benzpyrene, 0·3%. Lung. Day 143. × 26. 25/33.

FIG. 11—Methylcholanthrene, 0·06%. Right axillary gland. Day 230. × 108. 106/34.

PLATE 21

FIGS. 12–15—Tumours of connective tissue in rats receiving methylcholanthrene *sub cutem* in lard, fig. 8.

FIG. 12—Primary tumour. Day 144. × 144. LR 215.

FIG. 13—Autograft from tumour shown in fig. 12, made 16 days before death. × 400.

FIG. 14—Primary tumour showing giant cells. Day 191. × 144. LR 226.

FIG. 15—Primary tumour. Day 175. × 144. LR 222.

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The Supposed Coagulation of Oxalate Plasma by Trypsin

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In 1917 Heard concluded from experiments on the physical properties of proteins that the coagulation of blood is due to a physical modification of fibrinogen into fibrin produced by surface forces developed when the chemical affinities of bound Ca and P are brought into action. On this hypothesis he determined the capacity of solutions of trypsin to coagulate oxalate plasma, since that enzyme possesses a well-recognized power to attack organically bound phosphorus. He found that the power of a solution of trypsin to coagulate oxalate plasma was parallel to its capacity to dissolve coagulated albumen.

After discussing various possible explanations of the phenomenon, Heard concluded that trypsin under definite conditions is able to function as thrombase and regarded this fact as strong evidence in favour of his hypothesis of blood coagulation.

In this investigation solutions of thrombase, prothrombase, thrombokinase, phosphate plasma, trypsin and trypsinogen were used.

Thrombase and prothrombase were prepared by methods previously described (Mellanby, 1930, 1933). The strength of the solutions was 0·05%.

The solution of thrombokinase was prepared by extracting 10 gm of bull's testis with 100 cc of H_2O. The filtered extract was diluted with H_2O until activation of 1 cc of prothrombase (0·05%) containing 0·05% $CaCl_2$ was produced within 5 minutes after the addition of 0·1 cc of the kinase solution.

Phosphate plasma (Bordet and Delange, 1912) was prepared by shaking vigorously oxalated plasma to which one-third of its volume of a 5% suspension of colloidal calcium phosphate in water had been added. After 10 minutes' shaking the mixture was centrifuged and the clear plasma obtained.

The trypsin solution was prepared by dissolving 1 gm of commercial trypsin in 100 cc of H_2O.

The trypsinogen solution was prepared by extracting a fresh pig's pancreas with 0·1 HCl (N) at room temperature for 12 hours. The

extract, when neutralized, gives a clear filtrate containing a large quantity of trypsinogen but no trypsin or other enzyme of the pancreas (Mellanby and Woolley (1914)).

(*a*) THE COAGULATION OF OXALATED RABBIT'S PLASMA BY TRYPSIN

The rabbit's plasma contained $K_2\bar{O}x$ 0·1%.

The solutions given in Table I were made up and the times of coagulation observed.   All experiments were carried out at 38° C.

TABLE I

	Oxalated plasma	H_2O	Trypsin (1%)	Coagulation time
	cc	cc	cc	
(1)	1	0·9	0·1	No coagulation
(2)	1	0·8	0·2	50 seconds
(3)	1	0·7	0·3	45 seconds
(4)	1	0·6	0·4	1 minute
(5)	1	0·5	0·5	1·3 minutes
(6)	1	0·4	0·6	No coagulation

The results confirm Heard's original observations.

Experiments (1) and (6) are of interest.   In experiment (1) the amount of trypsin solution was too small to produce coagulation.   After 1 hour 0·1 cc of thrombase coagulated the solution in 10 seconds, indicating that the essential mechanism of blood coagulation was intact.   In experiment 6) no coagulation was produced and the subsequent addition of thrombase produced no clot—the fibrinogen had been digested by the excess of trypsin.   In the intervening experiments the proteolytic activity of the trypsin was annulled by the antitrypsin of the plasma so that the full coagulating activity of the trypsin on the fibrinogen was able to exert itself.

(*b*) THE COAGULATION' OF PHOSPHATE PLASMA BY TRYPSIN

Phosphate plasma contains fibrinogen but no prothrombase.   It does not clot on calcification but only on the addition of thrombase.

Solutions of phosphate plasma, kinase, prothrombase and trypsin given in Table II were made up and the times of coagulation observed.

TABLE II

Phosphate plasma	Kinase	Prothrombase (0·05%)	Trypsin (1%)	Coagulation time
cc	cc	cc	cc	
1	0·3	0	0	No coagulation
1	0·3	0	0·3	,,
1	0·3	0·3	0	,,
1	0·3	0·3	0·3	45 seconds

2 D 2

It is evident that trypsin solutions produce coagulation by acting on prothrombase and converting it to thrombase. Plasma free from prothrombase cannot be coagulated by trypsin. The results do not support Heard's assumption that trypsin acts as thrombase.

(c) The Activation of Prothrombase by Trypsin

A solution containing prothrombase, kinase, and trypsin was made up.

Prothrombase (0·05%)	Kinase	Trypsin (1%)
1 cc	0·3 cc	0·3 cc (X)

After varying terms 0·2 cc of X was added to 1 cc of oxalate plasma and the times of coagulation noted. After 5 minutes 0·2 cc of X coagulated the plasma in 12 seconds; before that time and 10 minutes later the solution X was unable to coagulate oxalate plasma.

It is evident that the trypsin solution converted the prothrombase to thrombase in 5 minutes, but after that time the trypsin digested the thrombase by means of its proteolytic properties.

(d) The Coagulation of Oxalated Plasma by Boiled Tryspin

Attempts were made to determine the conditions under which trypsin converts prothrombase into thrombase.

The relations of enterokinase to trypsinogen and thrombokinase to prothrombase suggested various possibilities on the relation of these two kinases to one another. But these speculations proved fallacious since solutions of trypsinogen, free from trypsin, possessed the property of coagulating oxalate plasma. Further, the generalization of Heard, that the capacities of solutions of trypsin to coagulate oxalate plasma run broadly parallel with their proteolytic activities, was not confirmed. Many weak solutions of trypsin readily clotted oxalate plasma; on the other hand, many trypsin solutions of pronounced proteolytic power were inactive. The problem, therefore, was reinvestigated on the assumption that trypsin was not the active coagulating agent.

A solution of trypsin was heated to 100° C for 1 minute and the coagulated protein filtered off. The clear filtrate contained no trypsin.

The coagulating activity of the filtrate is shown in the figures in Table III.

The results are the same as those shown in (a) except that 0·6 cc of the boiled trypsin solution produced coagulation, whereas the original

trypsin solution produced no coagulation in this quantity since it digested the fibrinogen in the plasma. These results indicated that a thermo-stable substance was present in the trypsin solution which produced the coagulating effects.

The trypsin powder was therefore ashed, the ash extracted with dilute hydrochloric acid, neutralized and made up to the same volume as the original trypsin solution. This solution coagulated rabbit's oxalate plasma in a manner comparable to that observed with the original trypsin solution. It was evident, therefore, that the active coagulating agent was an inorganic salt, and experiments indicated that calcium, although present in very small concentrations, was the active agent.

TABLE III

Rabbit's oxalated plasma	H_2O	Trypsin 1% boiled and filtered	Coagulation time
cc	cc	cc	
1	0·9	0·1	No coagulation
1	0·8	0·2	45 seconds
1	0·4	0·6	45 seconds

It became necessary, therefore, to carry out a series of quantitative experiments to determine what relation calcium bears to kinase in the coagulation of oxalate plasma. It has been established that the addition of a quantity of calcium chloride to the plasma in excess of the oxalate contained in it invariably produces coagulation, but whether quantities of calcium less than the equivalent of oxalate can produce that effect has not been determined.

(e) THE COAGULATION OF OXALATED PLASMA CONTAINING VARYING QUANTITIES OF KINASE BY CALCIUM CHLORIDE

The plasma was obtained from ox blood and contained $K_2\overline{O}x$ (N/40).

The results in Table IV show that the quantity of calcium required to coagulate oxalate plasma is determined by the quantity of kinase in the plasma. Plasma (1) containing little kinase required an excess of calcium over the oxalate contained in it; plasma (5) containing a large quantity of added kinase required a quantity of calcium equivalent to one-tenth of the oxalate present to produce coagulation.

The experimental results may be summarized:

Final concentration of oxalate in plasma—$K_2\overline{O}x$ N/80.

J. Mellanby

TABLE IV

Oxalate plasma	Kinase	H_2O	$CaCl_2$	Coagulation time
cc	cc	cc	cc	
(1) 1	0	0·9	0·1 $CaCl_2$ N/5	No coagulation
1	0	0·8	0·2 ,,	3 minutes
(2) 1	0·1	0·8	0·1 $CaCl_2$ N/10	No coagulation
1	0·1	0·7	0·2 ,,	1·5 minutes
(3) 1	0·2	0·7	0·1 $CaCl_2$ N/40	No coagulation
1	0·2	0·6	0·2 ,,	1·6 minutes
(4) 1	0·3	0·5	0·2 $CaCl_2$ N/80	No coagulation
1	0·3	0·4	0·3 ,,	1·3 minutes
(5) 1	0·4	0·3	0·3 Ca Cl_2 N/160	No coagulation
1	0·4	0·2	0·4 ,,	2 minutes
(6) 1	0·5	0·2	0·3 $CaCl_2$ N/160	No coagulation
1	0·5	0·1	0·4 ,,	2·4 minutes
(7) 1	0·6	0·1	0·3 $CaCl_2$ N/160	No coagulation
1	0·6	0·0	0·4 ,,	2·6 minutes

Relation of $CaCl_2$ to kinase to produce coagulation in approximately 2 minutes.

$CaCl_2$ N/50   N/100   N/400   N/550   N/800   N/800   N/800

Kinase added to
1 cc of plasma   0   0·1   0·2   0·3   0·4   0·5   0·6

The minimal quantity of $CaCl_2$ required to produce coagulation, however large the concentration of kinase in the oxalated plasma, is approximately N/800. This gives a concentration of 2·5 mgm of Ca in each 100 cc—a value about one-quarter of the calcium content of normal blood.

The figures show that oxalated plasma, relatively rich in kinase, may be coagulated by the addition to it of a quantity of calcium approximately equivalent to one-tenth of the soluble oxalate present.

The facts afford an adequate explanation of the phenomena observed in the coagulation of oxalated plasma by solutions of trypsin—coagulation being due to the calcium present in the trypsin solution acting in association with a large quantity of kinase in the oxalated plasma.

SUMMARY

Some solutions of trypsin coagulate oxalate plasma. The action is due to the conversion of prothrombase to thrombase by the ionized calcium contained in the trypsin solution.

The quantity of calcium required to coagulate oxalate plasma is determined by the thrombokinase content of the plasma. Plasma containing $K_2\overline{O}x$ N/80 and an optimal quantity of kinase may be coagulated by the addition of $CaCl_2$ N/800. This fact indicates the avidity of kinase for securing the calcium ions to the prothrombase-kinase system.

Mammalian blood collected directly into a solution of potassium oxalate obtains kinase relatively slowly from the cells of the blood. The interval of time after leaving the blood vessels is greater than that required for the precipitation of 75% of the total calcium of the blood by the potassium oxalate.

Solutions, tested for thrombase, should contain an excess of a soluble oxalate.

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Cellular Individuality in the Higher Animals, with Special Reference to the Individuality of the Red Blood Corpuscle—III

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Introduction

It has been shown in previous communications (Todd, 1930, *a*, *b*) that the red blood corpuscle of the domestic fowl constitutes a " multiple antigen " of considerable complexity and of so high a degree of specificity that, in unrelated birds, the red cells of any particular fowl can be differentiated from those of any other individual of the same species, by means of a simple agglutination reaction carried out with suitably exhausted polyvalent iso-agglutinating sera.   When the birds are closely related, however, this is not always possible, as the corpuscles of certain members of a family may show a very close resemblance.

In the course of some earlier experiments, made in order to investigate the hereditary transmission of the characters of the red blood corpuscle, three separate couples of unrelated Plymouth Rock birds were mated, and the cells of parents and offspring in each of the three families examined in detail (Todd, 1930, *b*).   On reviewing the results obtained and comparing the behaviour of the corpuscles of the individual members in any one family, it was seen that the cells of no two chicks appeared to be exactly alike, but that there was always some degree of difference, varying from a close resemblance to a very marked contrast; suggesting that the red cells contain a number of different antigenic units, each of which behaves independently during the process of hereditary transmission.

If this is true it seemed that it should be possible, by mating brother and sister birds specially selected as having closely similar corpuscles, to produce a strain the individual members of which would be serologically indistinguishable, or at any rate would possess red cells so much alike that differentiation would be difficult.

INBREEDING EXPERIMENTS ON BIRDS WITH " LIKE " CORPUSCLES

With this end in view four pairs of birds were chosen from the specially bred families of Plymouth Rock fowls referred to above, each pair consisting of a brother and sister whose red cells showed a very close resemblance. These birds were then mated and isolated in separate pens, any eggs laid being marked and incubated.

These matings were made in July, 1929, and considerable difficulties were met with before a continuously inbred (full brother-sister mated) strain could be established, as both the eggs and the chicks were at first very small and many of the chicks died before hatching. Finally three of the couples were discarded, and attention was concentrated on the remaining pair which was giving more promising results. This pair consisted of a cockerel (No. 34) and a pullet (No. 68) belonging to one of the families the character of whose corpuscles had already been investigated in detail (Todd, 1930, *b*). These results are reproduced here as Table I, and it will be noted that the cells of the two birds showed a remarkable similarity when tested by two different methods, and also that an immune serum exhausted for the corpuscles of either bird was exhausted for the corpuscles of the other.

Three generations have been reared from this couple by a series of successive brother-sister matings. The strain is now giving less trouble, and the eggs are becoming slightly larger and the texture of the shell better. There is still very slow feathering in the chicks, and up to the age of about 4 months they require more warmth than normal birds. Whereas the ordinary chicks are turned out at the age of about 3 months, the inbred chicks cannot be put out until about 4½ months old. This is probably due to the lack of feathers, which renders them more susceptible to cold. The male chicks are much lighter in colour than the female; this is also often the case with the ordinary Plymouth Rock chicks, but is not invariable, as it appears to be with the inbred chicks. There have been no black birds in this particular family. The pedigree of the family is given in Table II.

The corpuscles of the surviving members of this inbred family have been examined by means of a series of iso-agglutinating sera exhausted for other members of the same family, the tests being made on a rocking plate in the manner previously described (Todd, 1930, *a*).

A number of these tests are given in Table III, and it will be seen that a polyvalent iso-agglutinating serum exhausted for the cells of any one member of the inbred family (*i.e.*, the original " like " parents or any of their offspring down to the third generation) had practically lost its

TABLE I—EXAMINATION OF THE CORPUSCLES OF MEMBERS OF A FAMILY OF PLYMOUTH ROCK FOWLS (WHITE FAMILY) BY MEANS OF SPECIALLY EXHAUSTED ISO-AGGLUTINATING SERA

Immune serum exhausted with corpuscles of	Tested on corpuscles of																			
	F m.	M f.	19 f.	20 f.	22 m.	23 m.	24 f.	26 m.	27 f.	34 m.	36 f.	38 m.	39 f.	40 f.	41 f.	248	52 m.	53 f.	68 f.	69 f.
Father No.30	—	C.	—	C.	C.	—	a.c.	—	C.	C.	C.	C.	C.	—	C.	—	C.	—	C.	C.
Mother No.31	C.	—	+	+	C.	++	—	C.	—	—	—	+	—	C.	—	—	C.	C.	—	+
Chick No. 19	C.	C.	—	C.	C.	C.	C.	C.	C.	C.	C.	+	C.	C.	C.	+	C.	C.	C.	C.
20	C.	C.	C.	—	++	C.	C.	C.	—	C.	—	C.	C.	C.	C.	+	—	C.	C.	—
22	C.	C.	C.	—	—	C.	C.	C.	—	C.	C.	—	C.	C.	C.	C.	—	C.	C.	—
23	C.	C.	C.	C.	C.	—	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	—
24	+	—	C.	C.	C.	C.	—	C.	—	—	—	C.	+	C.	+	C.	a.c.	C.	C.	C.
26	C.	C.	—	C.	C.	—	C.	—	C.	C.	C.	C.	—	—	C.	—	C.	C.	—	+
27	C.	C.	C.	C.	C.	C.	C.	C.	—	C.	a.c.	C.	C.	C.	C.	—	C.	—	C.	C.
34	—	a.c.	C.	C.	C.	C.	—	C.	++	—	C.	—	C.	C.	C.	—	C.	C.	C.	C.
36	C.	C.	C.	a.c.	a.c.	C.	a.c.	C.	—	C.	—	C.	C.	C.	C.	C.	a.c.	C.	C.	C.
38	C.	C.	C.	C.	C.	C.	C.	C.	—	C.	+	—	C.	C.	C.	C.	C.	C.	a.c.	a.c.
39	C.	C.	—	C.	C.	C.	++	C.	C.	—	+	—	—	+	—	C.	C.	C.	C.	C.
40	C.	C.	+	C.	++	++	C.	—	C.	C.	C.	C.	C.	+	C.	+	C.	—	+	C.
41	—	—	—	C.	C.	C.	—	++	a.c.	—	C.	—	—	C.	—	+	C.	C.	C.	C.
248	C.	C.	a.c.	—	—	C.	C.	C.	C.	++	++	C.	C.	C.	C.	+	C.	C.	C.	C.
52	C.	C.	C.	C.	C.	C.	C.	C.	+	+	C.	—	C.	—	C.	—	—	C.	C.	C.
52	—	C.	—	C.	C.	++	C.	—	C.	C.	—	C.	C.	C.	C.	—	C.	—	—	C.
68	C.	C.	C.	C.	C.	+++	++	C.	a.c.	—	C.	++	C.	C.	C.	C.	C.	C.	—	C.
69	C.	C.	C.	—	+	C.	C.	C.	—	C.	—	—	C.	C.	C.	C.	—	C.	C.	—

agglutinating power for the cells of all other members, so that the corpuscles of all the members were serologically almost indistinguishable and, as regards the characters of its red blood corpuscles, the whole inbred family might be regarded as roughly resembling a single individual.

TABLE II—GENEALOGY OF THE INBRED " LIKE " FAMILY
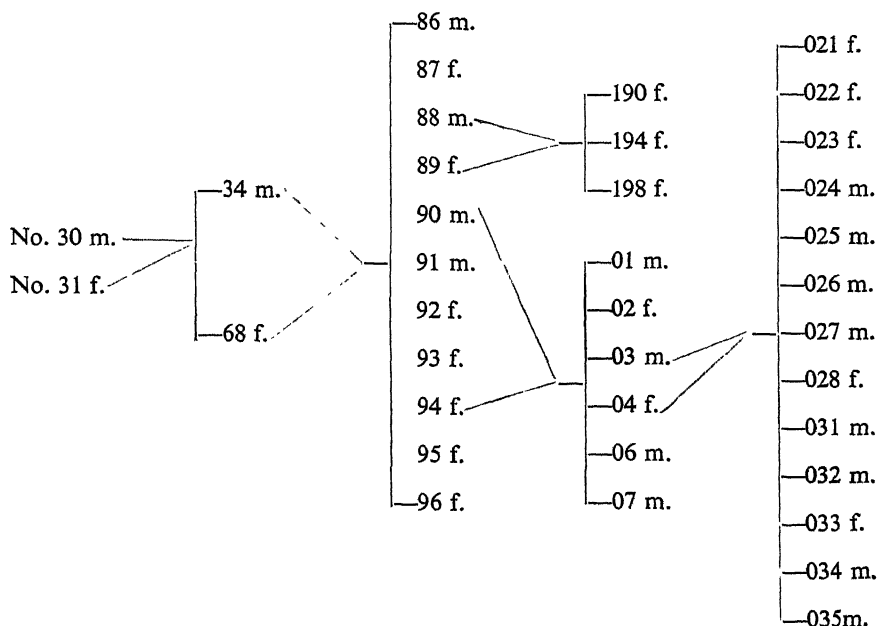
The originators of the family (Nos. 30 and 31) were presumably unrelated birds. The parents of each succeeding generation were full brother and sister, having closely similar red blood corpuscles.

In most tests the exhaustion appeared to be absolute and no trace of agglutination was observed, even on microscopical examination. In some of them, however, faint traces of agglutination could be seen microscopically, but these were quite minimal and not to be compared with the massive clumping present in the controls made with the cells of unrelated birds. In view of the fact that the corpuscles of fowls No. 34 and No. 68 (the originators of the inbred strain), although very similar, were not entirely alike, it was to be expected that some slight differences would be present in the cells of their descendants.

In a number of further experiments, iso-agglutinating sera exhausted with the corpuscles of different unrelated fowls were tested on corpuscles of the various members of the inbred family. With these sera, naturally, high degrees of agglutination were obtained but, for any one exhausted

serum, the amount of agglutination was approximately, though not always exactly, the same for all members of the inbred family.

It thus appears that it is possible, by the selection and mating of a pair of brother and sister fowls whose corpuscles are serologically closely similar, to produce a strain of birds in which all the individual members, at any rate as far as the third generation, show quite a remarkable resemblance in the behaviour of their red corpuscles to iso-agglutinating sera. Such inbred strains may possibly prove to be of value in certain genetic investigations, and the strain described has already been utilized in the experiments recorded below.

TABLE III—TEST OF THE CORPUSCLES OF MEMBERS OF THE INBRED FAMILY, WITH ISO-AGGLUTINATING SERUM EXHAUSTED WITH THE CORPUSCLES OF VARIOUS MEMBERS OF THE SAME FAMILY

Tested on corpuscles of fowl No. :—	Immune iso-agglutinating serum 15/14 exhausted with corpuscles of fowl No. :—								
	26	31	2	4	6	7	68	23	33
31	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0
33	0	tr	0	tr	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	tr	0	0	tr	0	0	0	0
25	0	tr	0	0	tr	0	0	0	0
26	0	tr	0	0	tr	0	0	0	0
27	0	tr	0	0	tr	0	0	0	0
28	0	0	0	0	0	0	0	0	0
02	0	tr	0	0	tr	0	0	0	0
04	0	tr	0	0	tr	0	0	0	0
06	0	0	0	0	0	0	0	0	0
07	0	tr	0	0	tr	0	0	0	0
Unrelated control B	C.4½	C.2	C.11	C.3½	C.3	C.8	C.4	C.4	C.3
Unrelated control C	C.3½	C.2	C.6½	C.5	C.3	C.4	C.3½	C.4	C.3

C.4½ = complete agglutination in 4½ minutes.
tr = trace of agglutination (microscopical).

IS IT POSSIBLE TO RECOGNIZE ANY SEROLOGICAL DIFFERENCE BETWEEN THE RED BLOOD CORPUSCLES OF MALE AND FEMALE FOWLS ?

The fact that differences between the corpuscles of individual fowls are so easily demonstrated, by the aid of serological methods, suggested that

it might be possible, by the use of these methods, to detect a difference between the corpuscles of the two sexes, if any such difference existed. Experiments were carried out in order to examine this point.

In view of the fact that in the fowl it is the female which is the hetero-gametic sex, it was thought advisable to use female corpuscles for the preparation of the sera, which were made both in male and in female birds.

A number of experiments, of which the following is an example, were carried out in this way. Three cocks were immunized with hens' blood, each bird receiving intramuscular injections of 20 cc of the blood at weekly intervals for 9 weeks. As each bird had received the blood of only nine individuals, the sera were naturally not very polyvalent, but one of the cocks gave a serum which was reasonably so, and this serum was used for the tests.

The serum was first exhausted with the corpuscles of three cocks and the resulting exhausted serum, when tested on the cells of male and female birds, behaved as follows:—

> tested on the corpuscles of 8 cocks—agglutinated 3 out of 8;
> tested on the corpuscles of 7 hens—agglutinated 3 out of 7.

Another portion of the same serum was then exhausted with the corpuscles of eight cocks, after which it gave the following result:—

> tested on the corpuscles of 4 cocks—agglutinated 1 out of 4;
> tested on the corpuscles of 7 hens—agglutinated 2 out of 7,

showing that, even after exhaustion with the corpuscles of eight male individuals, the relative agglutinating power of the serum for the cells of the different sexes was not materially altered.

Other experiments gave similar results and made it clear that, generally speaking, and in unrelated fowls, an antiserum prepared by immunizing cocks with the corpuscles of an adequate number of hens will agglutinate, in varying degree, the corpuscles of most individual fowls—independently of their sex. After exhaustion with the corpuscles of one cock the serum will agglutinate the corpuscles of a smaller number of individuals. Further exhaustion with the corpuscles of a second cock again reduces the number of individuals whose corpuscles are agglutinated by the exhausted serum, and so on. Experiment shows that even after exhaustion with the corpuscles of as many as eight individual cocks the serum may still be active for the corpuscles of certain individuals, and it does not seem to be significantly more active for the cells of hens than for those of cocks.

This being so, it was obvious that investigation along these lines was unlikely to yield a decisive answer, until some means of eliminating the dominating individuality reactions of the corpuscles could be found.

This difficulty was ultimately overcome by means of the inbred family described above, the red cells of whose members were serologically practically indistinguishable, so that the question of individual reactions did not arise.

The iso-agglutinating serum used for the tests consisted of the pooled sera of eight Plymouth Rock hens, each of which had received six intra-musclular injections (approximately 140 cc) of citrated fowls' blood. The injected blood was from 25 different individuals of various breeds—all except two being hens—so that the resulting serum was highly poly-valent.

Separate portions of this serum were exhausted four times with (a) corpuscles of fowl No. 07, a *male* of the inbred family of " like " birds; (b) corpuscles of fowl No. 68, a *female* of the inbred family of " like " birds. The two exhausted sera were then tested on the cells of male and female members of the same family. The results, given in Table IV, were quite sharp, no trace of agglutination being given by the corpuscles of either sex.

TABLE IV—TEST OF THE CORPUSCLES OF MALE AND FEMALE INBRED BIRDS WITH ISO-AGGLUTINATING SERUM EXHAUSTED WITH MALE AND FEMALE CORPUSCLES

Tested on corpuscles of fowl:—	Iso-agglutinating serum 105/15 exhausted with *male* corpuscles from fowl No. 07	Iso-agglutinating serum 105/15 exhausted with *female* corpuscles from fowl No. 68
Male, No. 06	0	0
„ „ 07	0	0
„ „ 25	0	0
„ „ 26	0	0
„ „ 27	0	0
Female, No. 21	0	0
„ „ 22	0	0
„ „ 23	0	0
„ „ 28	0	0
„ „ 04	0	0
Unrelated control A	Complete (4 min)	Complete (5 min)
„ B	Complete (11 min)	Complete (15 min)

A repetition of the test, with double volumes of the exhausted sera, and prolongation of the time of rocking of the plate on which the

agglutinations were made from 15 to 30 minutes, produced no change in the result.

We must therefore conclude that, as regards their behaviour towards iso-agglutinating sera and apart from their individual characters, no difference between the red blood corpuscles of male and female birds can be detected.

<center>DISCUSSION</center>

The results of an examination of the corpuscles of a number of inbred fowls have recently been recorded by Kozelka (1933), who employed mono- and polyvalent hetero-agglutinating sera prepared in the rabbit, and who used the technique employed by Landsteiner and Miller (1924). Kozelka apparently did not study the characters of the cells of the parents and offspring in any particular inbred family, but confined his investigations to an examination of the corpuscles of a large number of members of several strains with different coefficients of inbreeding. He found that the individual members of an inbred strain might show wide degrees of difference in the character of their corpuscles, and notes one strain in which, in spite of inbreeding equivalent to almost three generations of full brother-sister matings, no two individuals were identical in their agglutinogens. Still, in all the experiments in which the individuals of one inbred strain were tested with those of another strain, there was a general differential reaction in the two groups. There was also a significant indication of similarity with respect to the agglutinogens within a group of inbred individuals, showing that the inbreeding brings about a certain degree of similarity of the agglutinogens.

As we know that the first generation derived from parents with dissimilar corpuscles may show a wide range of individual differences in their corpuscles, it is clear that random mating between members of this generation will result in individual differences in the progeny. As, however, the cells of the offspring of any mating, whether inbred or otherwise, can have no agglutinogens not contained in the cells of their parents, the agglutinogens present in the corpuscles of all the members of any wholly inbred family must necessarily be limited to those present in the original founders, and in this sense the cells must possess a certain degree of similarity.

On the other hand, when the mating is selective and is made between birds with closely similar corpuscles, the cells of the individual members of the resulting inbred family, as seen in the experiments described above, may show a very close resemblance, the degree of which is presumably determined by the degree of resemblance between the cells of the selected

originators of the family. It is interesting to note that Kozelka describes a small inbred family in which the cells of the individual members possessed a high degree of similarity. No data are given regarding the corpuscles of the parents, but one would suppose that these probably showed a similar resemblance.

It is a pleasure to acknowledge my indebtedness to the Medical Research Council, to Captain S. R. Douglas, F.R.S., in whose department of the Institute the work was carried out, to Major G. W. Dunkin, M.R.C.V.S., and especially to Miss E. Salmon to whose skill and care the successful rearing of the inbred strain must be attributed.

Conclusions

If two unrelated domestic fowls are mated, the red blood corpuscles of the individual members of the first generation of their offspring show immunological differences, which may vary from a close resemblance to a very marked contrast; but by a selective brother-sister mating of members having closely similar corpuscles it is possible to produce a strain of birds in which the red cells of all the individual members show a very close resemblance, the degree of which is presumably determined by the degree of resemblance of the selected originators of the inbred family.

Such a strain has been carried through three generations of pure brother-sister matings, and the corpuscles of all the surviving members still retain their immunological similarity.

No difference between the red corpuscles of male and female birds could be detected by means of their behaviour towards exhausted iso-agglutinating sera.

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The Brain of *Gadus*, with Special Reference to the Medulla Oblongata and its Variations according to the Feeding Habits of different Gadidæ —I

By H. Muir Evans

Introduction

In a previous paper (Evans, 1932) we have described the medulla oblongata of the Cyprinidæ and have shown that the pattern of the medulla varies according to the habits of feeding, so that it was possible to divide the Cyprinidæ into four groups. We propose to attempt a similar study of the Gadidæ and it will be found that the pattern varies in a definite manner according to the methods of feeding, and the character of the food.

In carrying out this research we are very much indebted to the exhaustive examination of the stomach contents by Cunningham (1896) and more recently by Borley and Thursby-Pelham (1926). Before describing the medulla oblongata in the various members of the Cod family, it is necessary to point out the marked difference in the naked eye appearance of the hind-brains of the Cyprinidæ and Gadidæ. In Cyprinidæ the brain is characterized by the great development of the vagal and facial lobes. The vagal lobes form a pair of large swellings on either side of the rhomboid fossa and embrace between their anterior ends the unpaired facial lobe—a rounded body situated in mid line behind the cerebellum. This " lobus impar " represents apparently " a fusion of the two small facial lobes seen in the Cod " (Goronowitsch, 1897). The dorsal surface of the medulla in the Cod is occupied by a series of swellings that almost entirely close the rhomboid fossa.

Burne (1902) gives a good drawing of the brain of *Gadus morrhua* dissected, and states " the prominent lateral crura of the cerebellum contain besides cerebellar tracts a pair of large nuclei from which arise the acoustic and lateral-line nerves. These lobes apparently represent the tuberculum acusticum of Elasmobranchs. . . . Close behind the cerebellum and exposed by moving forwards its posterior extremity are a pair of prominent lobes (posterior crura of the cerebellum). . . . Behind these on a level with the exit of the vagus from the brain are a

pair of lobes almost contiguous in the middle line and each indistinctly divided by a longitudinal furrow. They give origin to the sensory roots of the facial and glosso-pharyngeal. Behind and to the sides of these lobes are elongated and somewhat irregular excrescences from which arise the sensory roots of the Vagus." This description follows the observations on the brain of *Lota* by Goronowitsch (1897).

We propose to give a series of typical sections of the hind-brain of the Whiting and compare them with sections at similar levels in the Roach.

For the purpose of our naked eye description of the various types of medulla in the Gadidæ it is suggested that the pair of prominent lobes (posterior crura of cerebellum) be described as the somatic sensory or lobi trigemini. The lobes that they partially embrace we shall call the facial lobes, as the glossopharyngeal element is only clear microscopically, so that for comparative study we shall speak of somatic sensory lobes, facial lobes, and vagal lobes from before backwards. Herrick (1907) criticizes the views of Goronowitsch (1897), Haller (1896), and Burne (1902) and disagrees in their recognition of a facial lobe. He states definitely that " in the Cod there is no well-defined facial lobe. Gustatory fibres from the mouth and from the outer skin terminate in the vagal lobe. The vagal lobe of the Cod is internally divided by a longitudinal septum into median and lateral lobules of about equal extent. The median lobule is primarily visceral and receives the gustatory fibres of the IX and X nerves. The gustatory root of the VII, which carries practically all the fibres from the taste buds in the outer skin and a smaller number from the anterior part of the mouth, terminates in both lobes but chiefly the lateral one. It is clear that the lateral lobule is primarily if not exclusively the terminal nucleus of the gustatory fibres of the somatic type."

To those whose early training has led them to take human anatomy as their starting-point in their introduction to neurology it is a little difficult to appreciate at first the terminology used in describing the nerves and nerve centres in fish. The subject can best be understood by considering " the components of the cranial nerves in which both the somatic sensory systems and the visceral motor is divided into two parts. The general somatic system carries impulses to the brain of touch, pain, gustatory, and other impulses; the acoustico-lateral portion conveys to the brain impulses of hearing and the equilibrial sense from the ear and lateral line sense organs " (Saunders and Manton, 1931). It is not now necessary to enlarge on the visceral motor system. Entering the medulla anteriorly in the Whiting is to be seen a large nerve which consists mainly of V and VII and VIII nerves. " They are made up of fibres of the

general somatic sensory system including the superficial ophthalmic of the VII and the V and the maxillary of the V nerve. All the fibres of these nerves are derived from nerve cells in the dorsal part of the medulla. The acoustico-lateral system is also represented in the VII, superficial ophthalmic, VII inner and outer buccals and VII external mandibular. The fibres of all these nerves terminate in the acoustico-lateral area of the medulla."

A diagram of the brain in "An Introduction to Neurology " by Herrick (1928), see fig. 1, illustrates the gustatory and acoustico-lateral systems of the fish Menidia. The gustatory system is present (indicated by cross-hatching) in VII, IX, and X cranial nerves, and to a less extent in the V, and all of these fibres, together with other visceral fibres enter the visceral
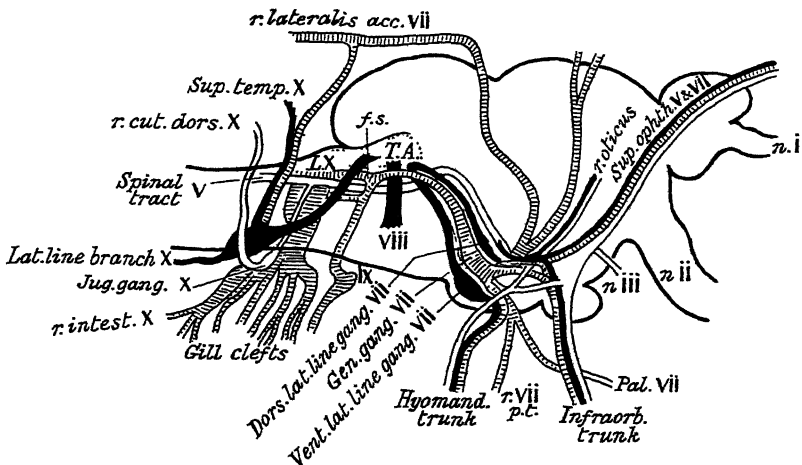
FIG. 1

sensory area in the vagal lobe (lobus vagi LX). Similarly the lateral line components of the VII and X nerves and the VIII (printed in solid black) converge to enter the acoustico-lateral area (formerly called the tuberculum acusticum-T.A.).

The general cutaneous fibres (somatic sensory) are outlined with finer lines and they enter the spinal V tract. In the diagram the brain is out-lined by heavy black lines. A careful inspection of the diagram shows the dorso-lateral line ganglion of the VII, the geniculate ganglion of the VII and the ventral lateral line ganglion of the VII. It will be seen that the superficial ophthalmic contains fibres of V and VII nerves and includes fibres belonging to the gustatory system, acoustico-lateral system and general cutaneous fibres (somatic sensory). Both the hyomandibular trunk and infraorbital trunk also contain fibres belonging to the three

systems. The pretrematic branch of the VII and palatine branch of the VII contain fibres belonging to the gustatory system.

In the same work by Herrick is a diagram on p. 295 of the cutaneous gustatory branches arising from the geniculate ganglion of the facial nerve of the catfish (*Ameiurus melas*). The gustatory system is well developed especially the vagal part which supplies taste buds in the gill region. Moreover, in some species taste buds appear in great numbers in the outer skin and these are all innervated by the VII nerve. These sense organs are entirely independent of the lateral line sensory system.

Before considering the variations of the pattern of the medulla oblongata in different members of the cod family we must recall the observation of Herrick, on *Ameiurus* and various gadoids, who showed that either tactile or gustatory stimuli alone may be correctly localized by the outer skin. The distribution of the nerves of touch have been investigated and the innervation of the cutaneous taste-buds have been studied in *Ameiurus*, *Gadus* and *Carassius*. The nerves of the cutaneous taste buds spring from the communis root of the facial nerve. The same facial root supplies taste buds in the anterior part of the mouth. In the Siluridæ and Cyprinidæ, the nerves supplying the taste buds in the outer skin, lips and palate terminate in a single nucleus which forms a dorsal pro-tuberance on the medulla oblongata, the facial lobe, while the taste buds of the pharynx and gill region are innervated from the vagal lobe farther back in the medulla oblongata (Herrick, 1907). This author states that in the cod, the facial lobes do not exist in the form described by earlier writers; he notes that in the gadoids, particularly the tom-cod and the hake, the free filiform rays of the pelvic fin function in a similar fashion to the barblets of *Ameiurus* and are likewise richly supplied with end organs of both taste and touch. In both the cod and pouting terminal buds have been described on the lips, barbel, pectoral fins, and body.

DESCRIPTION OF SERIAL SECTIONS IN WHITING

We have endeavoured to settle the question of the existence of a true facial lobe or lobes in Gadidæ by making a detailed examination of serial sections of the medulla oblongata of the whiting. We have made drawings of these sections at intervals and the study of fifteen drawings leads us to the definite conclusion that there is a true facial lobe, bilateral, in the whiting. Our opinion is rendered the more probable from the fact that the naked eye appearance of the medulla had led us to the erroneous supposition that the facial lobes were vagal lobes and the anterior lobes were facial lobes as their appearance suggested these lobes

as they exist in the Siluroids.   The sections were compared with sections of the medulla of the roach at corresonding levels.   The roach was chosen as an example of the Cyprinoids, in which group the fish is largely a " sight " feeder, a fish that will take the dry fly and not a mudfeeder, as carp or bream, or a ground feeder like the barbel or gudgeon.   The
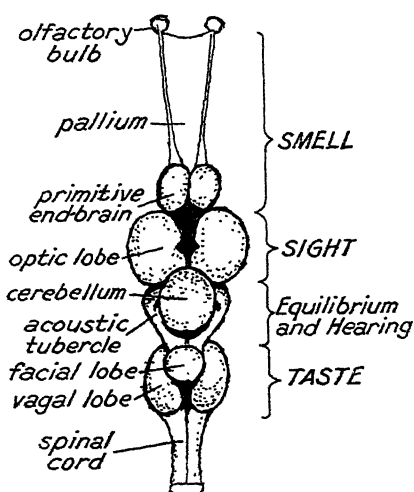
Fig. 2
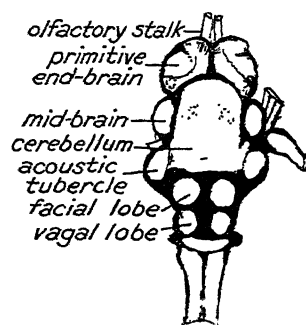
Fig. 3
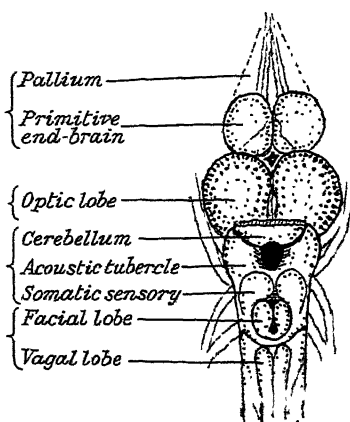
Fig. 4

whiting has no barbel. and its diet is almost entirely on other fish, in fact it feeds by sight.

Figs. 2, 3, and 4 are semi-diagrammatic drawings of the dorsal aspect of the brains of a carp, catfish, and cod.   In fig. 2, carp, the large vagal lobes are seen partially embracing the central facial lobe anteriorly.

In fig. 3, catfish, the bilateral facial lobes are seen lying immediately anterior to the somewhat smaller vagal lobes.

In fig. 4, cod, the posterior crura leading backwards from the tuberculum acusticum are seen to unite medially, and then form two globular prominences which approach each other in the middle line; posteriorly, these lobes are prolonged by a tail which partially surrounds the central facial lobes. These lie therefore at a deeper level and they unite at their hinder end. Behind these, again, are the vagal lobes.

These globular prominences which we have termed in the diagram the somatic sensory lobes, might have been thought at first sight to represent the facial lobes of the catfish, which older writers called " lobi trigemini." We shall see that this term might correctly be applied to them in the cod, as they consist almost entirely of a V nerve element.

Microscopic Structure of the Tuberculum Acusticum and the Somatic Sensory Prominences in the Whiting.—Fig. 5 is a section of the medulla on a level with the line pointing to the acoustic tubercle in fig. 4. To the left of the drawing the roots of the V, VII, and VIII nerves are seen passing into the medulla; in the most dorsal position are seen fibres passing into the acoustic tubercle which is just commencing to show its characteristic structure; next appear fibres of the V and VII nerves; the VII nerve has the characteristic position of the communis facial as seen in the carps and catfishes; it passes transversely inwards, and on the right side it is seen to turn sharply backwards so that it appears in part as cut transversely. On the left side and ventral to the VII nerve is seen the main trunk of the V nerve. On the right the combined trunk of these nerves is shown bearing ganglionic areas. Superiorly the cerebellum is shown in cross-section with its central granular area surrounded by an area containing cells of Purkinje which separate it from the molecular area. A molecular area is also present in the posterior crura and beneath this layer is an area which consists of a meshwork of cells and fine fibres.

Fig. 6 is a high power drawing of the dorsal portion of this area. It is enlarged from the section shown in fig. 8a where the crura have joined medially. Most dorsal is a molecular layer of very fine fibres within the meshes of which are a few small round cells. Beneath this is a layer of small round cells interspersed with larger round cells and other large cells either pear-shaped or bipolar. The whole is supported by a meshwork of fine fibres which surround groups of these cells and are finally prolonged into a bundle of nerve fibres which pass ventrally to decussate below the ventricle. In fig. 8a they appear as a broad bundle of fibres passing on the outer side of the facial roots.
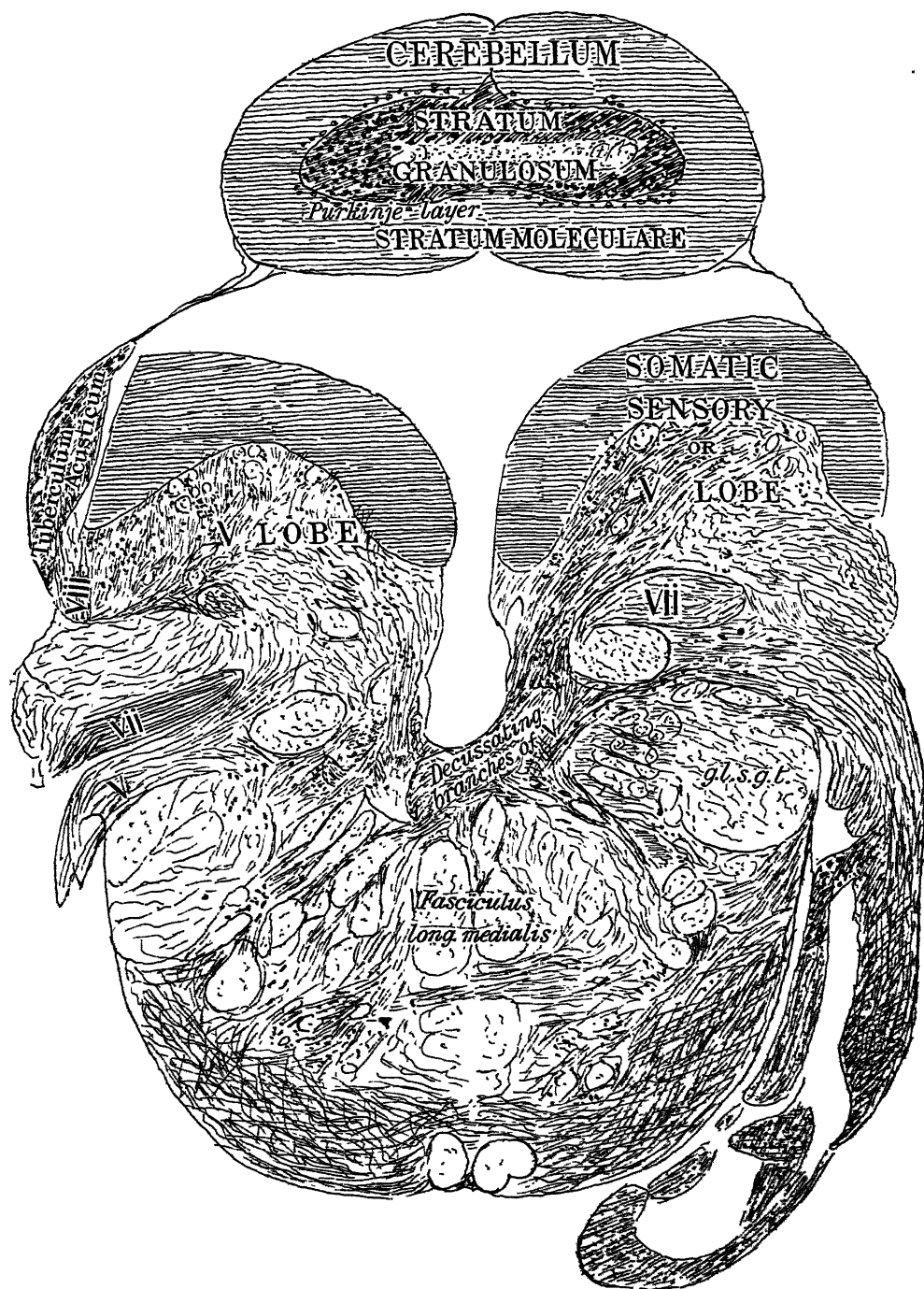
Fig. 5

Fig. 7 is anterior in position to fig. 5. It shows the distribution of the acustico-lateralis fibres passing into the acoustic tubercles. It is at a level where the lobi inferiores are fully formed, but the dorsal margins of these lobes are only indicated. The acoustic tubercles are fully formed, but only the ventral portion is shown and the cerebellum is also omitted. This section is shown to indicate the large motor areas here present.

As this section is cut slightly obliquely, the left side is rather more caudal than the right. The acoustic tubercle shows layers of closely packed round cells on the periphery; towards the internal margin these layers are broken up by nerve fibres running at first transversely inwards,
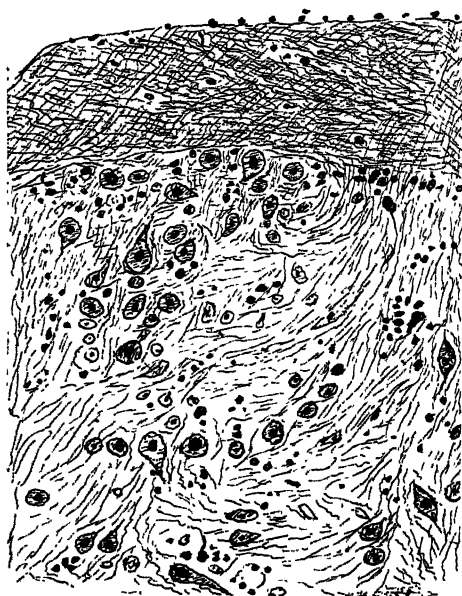
FIG. 6

and then turning ventrally as they approach the inner border of the tubercle. These fibres are at first bordered by interrupted chains of single rows of small cells, which become more scattered internally. The afferent nerve trunk is seen entering ventrally, and it breaks up into fasciculi which lie on the margin of the area of small round cells.

On the right side the grey matter of the molecular layer is seen to contain a meshwork of long separate fibres which pass into the ventral layer of spindle and pear-shaped cells interspersed with round cells from which fibres proceed to form a meshwork, terminating in a large bundle of fibres on the inner margin which passes ventrally. These

bundles decussate in the middle line in part, but they also terminate in a group of large motor cells, the fibres of which pass outwards and end into a descending longitudinal tract on either side.

Fig. 8*a* is a section at the level of the line pointing to the somatic sensory area in a whiting, fig. 4. The molecular superficial area is now very narrow. Under high power it appears as in fig. 6. The facial roots
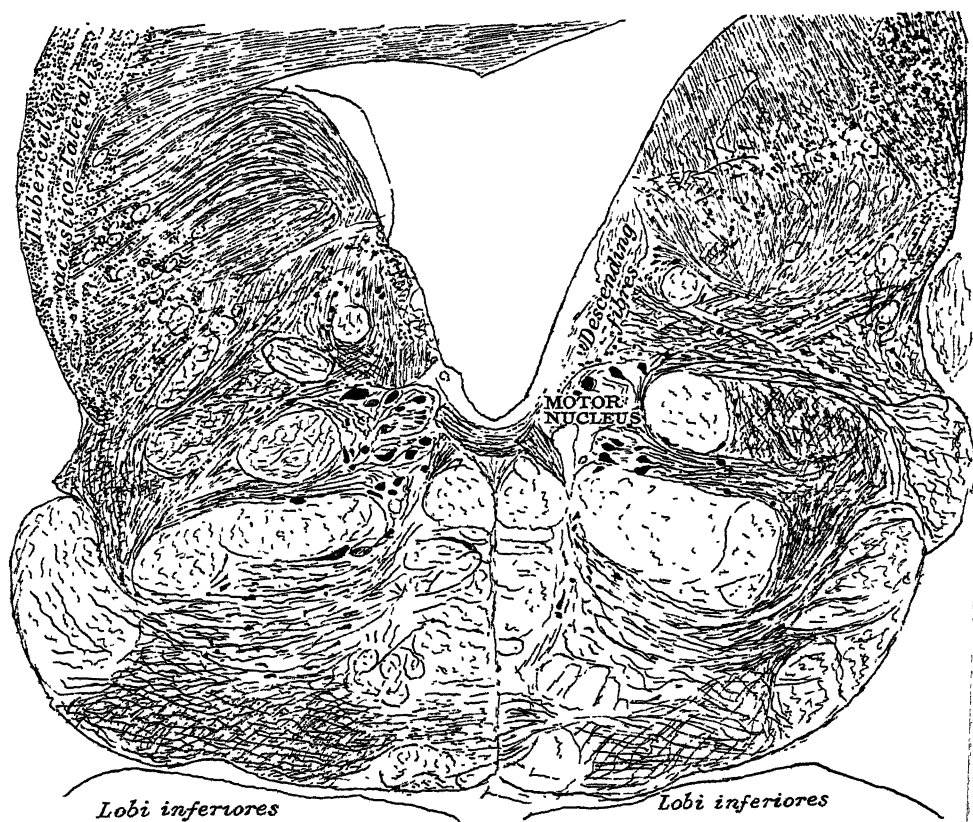
FIG. 7

appear cut transversely on either dorsal aspect of the ventricle. On the left, the V nerve fibres are seen entering the large globular eminences. From the meshwork of fine fibres occupying the centre of the lobes two large bundles of fibres are seen passing ventrally; the median longitudinal fasciculus gives off fibres ventrally, and these together with the descending bundles enter a central area of large motor cells.

These are arranged in a more ventral position than the motor cells described as connected with the descending fibres from the acoustic

tubercles (cf. fig. 7), and there is a considerable distance (200 serial sections) between the two motor areas. The fibres from these motor cells decussate and pass laterally to enter a special longitudinal tract.

We conclude from a study of these sections that the area in fig. 4
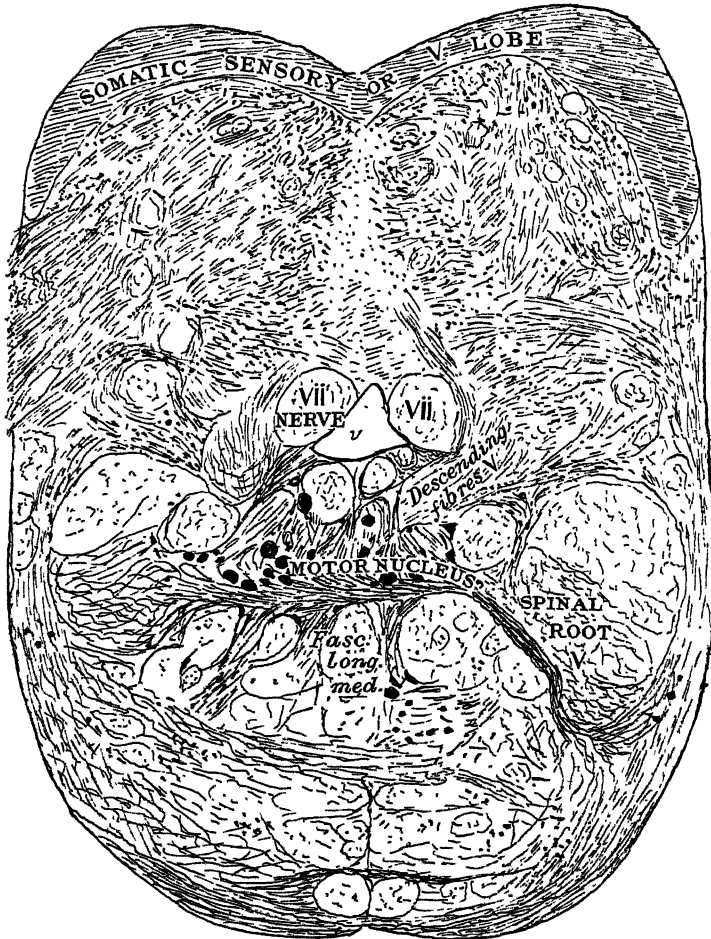
FIG. 8a—Whiting

termed somatic sensory is associated entirely with the V nerve and its sensory nuclei; these lobes may, therefore, correctly receive the name lobi trigemini, the term old authors applied to the facial lobes in the cat-fishes which occupy a similar position in the medulla oblongata. Another important fact to be noted is the presence in the so-called crura of two motor areas, one connected with the acustico-lateralis system and the

other with the V nerve. These areas are separated by some 200 serial sections, so that they are 1·2 mm apart.

This detailed description of the somatic sensory area is rendered necessary because the object of the paper is to call attention to the variation in size and pattern of these lobes, in relation to the habits of feeding of
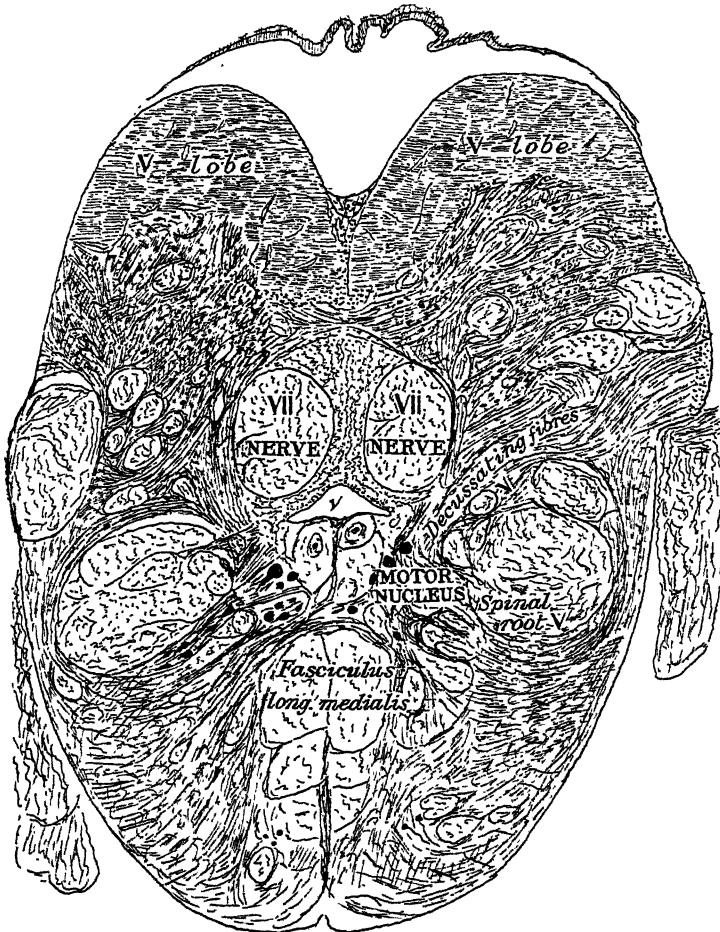
FIG. 8*b*—Roach

the various Gadidæ. The facial lobes vary in an inverse way as regards size with these lobes. But as the existence of these lobes has been doubted, it is now necessary to make a more detailed study of the hinder part of the medulla.

Fig. 8*a*, whiting, and fig. 8*b*, roach, are strikingly similar. In both the grey matter of the tuberculum acusticum covers an area of small cells

and terminal fibres of the V nerve, but this area is much broader in the whiting than in the roach. On either side of the ventricle and posterior to it are seen the gustatory fibres of the facial nerves cut transversely. These are larger in the roach than in the whiting. The descending decussating fibres of the V nerve are seen crossing ventral to the ventricle and terminating in large motor cells. These are more prominent in the whiting than the roach.
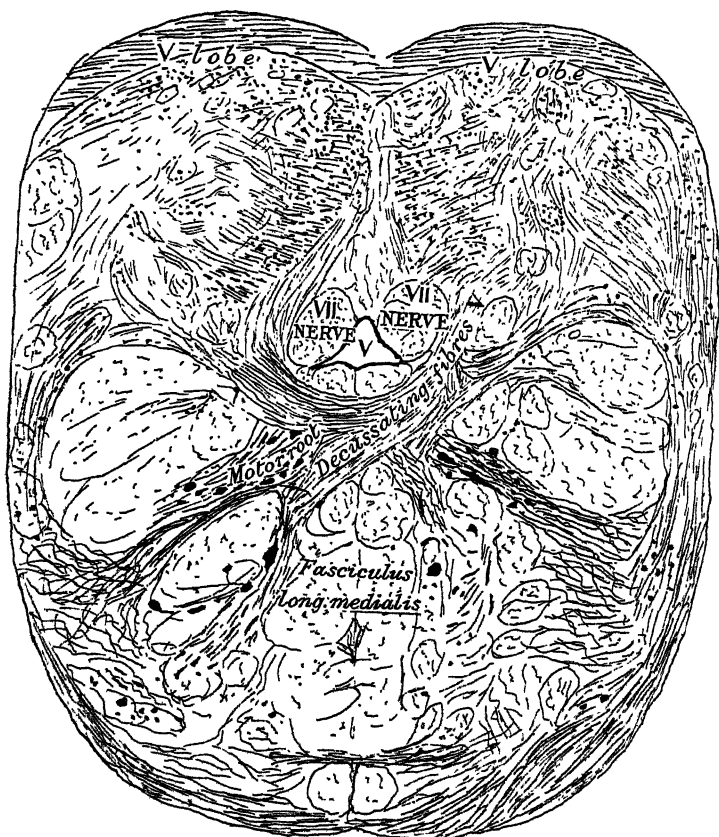
FIG. 9a—Whiting

Fig. 9a, whiting, and 9b, roach, are from sections posterior to fig. 8 a, b. In fig. 9b the facial lobe appears as a central ovoid area with groups of small cells characteristic of this lobe and between it and the ventricle on either side are seen facial trunks breaking up into bundles before entering the substance of the lobe. The descending fibres of the V nerve are shown and the great longitudinal gustatory tracts.

In fig. 9a the facial lobe has not appeared, but the small cellular structure of the V nuclei and its fibres form a prominent mass surmounted by the

grey matter continuous with the tuberculum acusticum. Near the ventricle on its dorsal aspect are seen the facial trunks breaking up into bundles.

Fig. 10*a*, whiting and fig. 10*b*, roach, are still further posterior. In fig. 10*b* the V lobe and its descending fibres are seen. The facial nerves
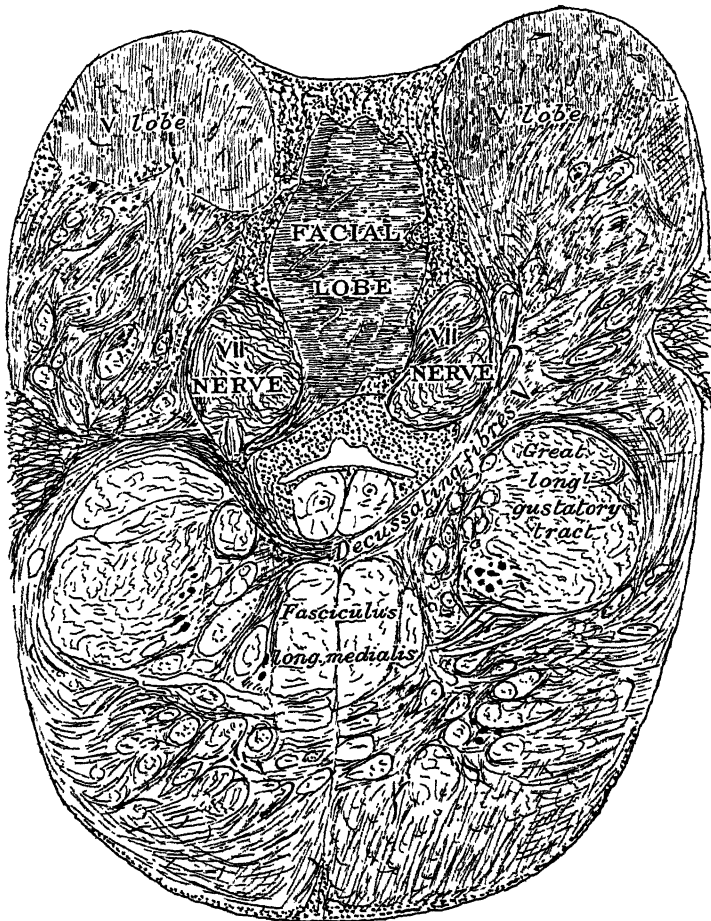
FIG. 9*b*—Roach

have entered the central facial lobe and descending fibres from it are seen entering the descending secondary gustatory tract. In fig. 10*a* the facial trunks are seen breaking up into small bundles in the midst of the anterior end of the facial lobes which first appear as a bulge towards the rhomboid fossa. The descending fibres of the V are seen decussating, and several bands are seen passing from the V lobe.

Fig. 11. *Whiting*—The facial lobes are now clearly seen and within its substance the fasciculi of the VII nerve. Descending fibres are seen passing from it into the great longitudinal secondary gustatory tract. The V lobe and its descending fibres are still evident.

Fig. 12. *Whiting*—The facial lobes are now a considerable size. Their descending gustatory fibres are seen entering the gustatory tract.
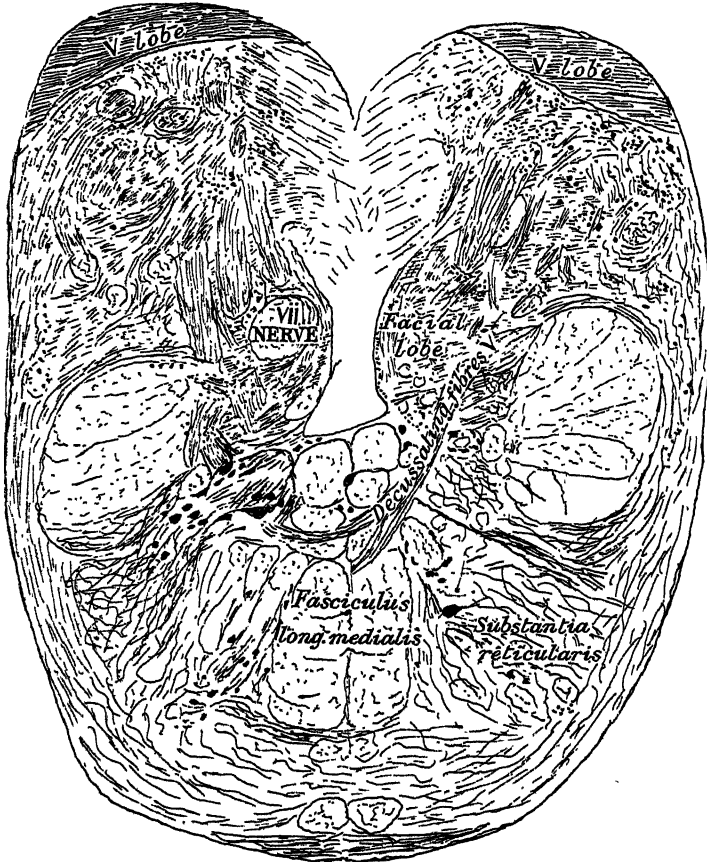
Fig. 10a—Whiting

Decussating fibres of the V are still seen, but the V lobe is becoming less evident.

Fig. 13—The facial lobes are becoming smaller and a division appears dorsally separating them from the glossopharyngeal lobes which now make their first appearance. On the right also the vagal nerve commences to appear.

Fig. 14. *Whiting*—The facial and glossopharyngeal lobes are still present. The trunk of the IX nerve appears, and entering transversely on the right is the X nerve; a small portion of the X also appears on the left.

Fig. 15—A portion of the VII lobe still appears dorsally, but the glosso-pharyngeal lobes are smaller. The nucleus ambiguus appear on both sides.
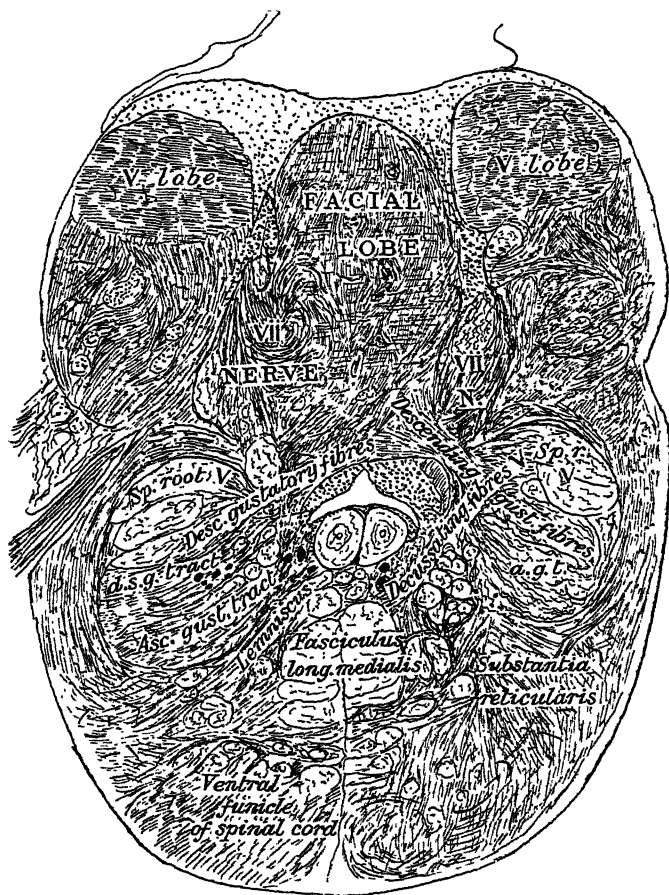
Fig. 10*b*—Roach

Fig. 16*a*—This section is taken still further caudal. It will at once be evident, when it is compared with the drawing at a similar level, fig. 16*b*, in the roach that the vagus lobi now occupies the dorsal part of the section. Both form and structure are similar in both roach and whiting. The section of the latter, however, still shows part of the glossopharyngeal lobe. The dorsal extremities of the vagals in the whiting approximate in the middle line. Both sensory and motor roots are well defined.

Fig. 17—Is the last of the series that it is necessary to examine. The vagal lobes are still evident, but they are now completely united dorsally. There is a large transverse strand, the dorsal commissure, extending across their united extremities. The nucleus ambiguus is still present.

It may seem unnecessary and tedious to discuss and picture so many sections. But it is only by this method that it is possible to establish
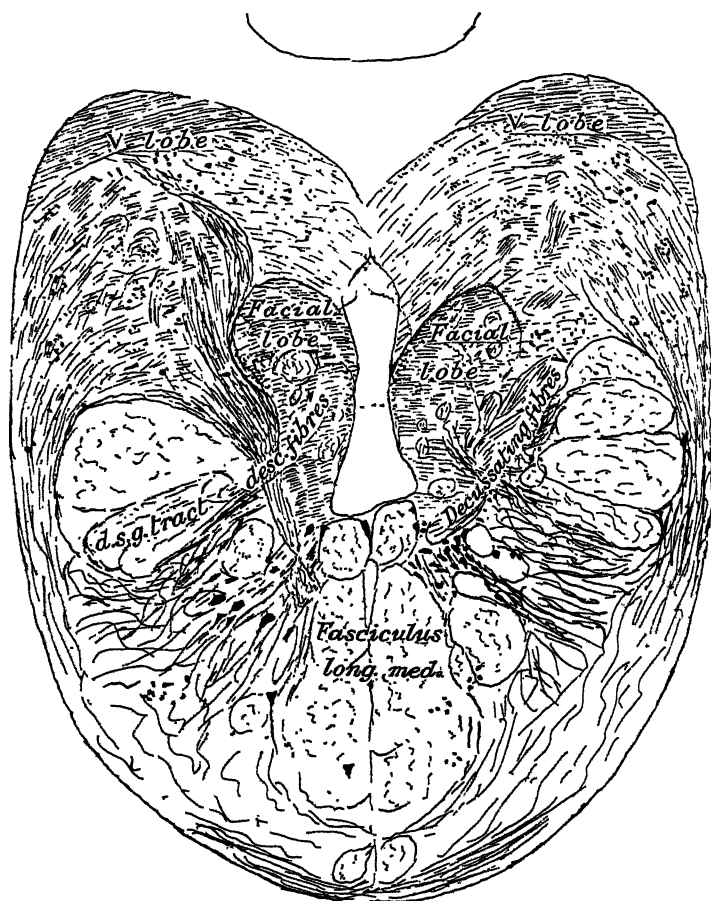
FIG. 11

definitely the accuracy of an opinion and render it an incontrovertible observation. It is clear if the above sections are studied that the brain of the whiting only differs in detail from that of a Cyprinoid. The essential element of a Teleost brain are present in both, and there is no need to dismiss a true facial lobe from the picture and elaborate a theory on a condition that has not been confirmed.

It appears to us that an important consideration in the identification of the various lobes rests not only on their relation to the nerve trunks and the various tracts with which they are connected but also on their histological structure. From the examination of a very large number of serial sections of the hind-brains of both Cyprinoids and Gadoids it is clear that each lobe has its characteristic arrangement and type of cell. It is thus possible to identify not only the vagal, the V and the
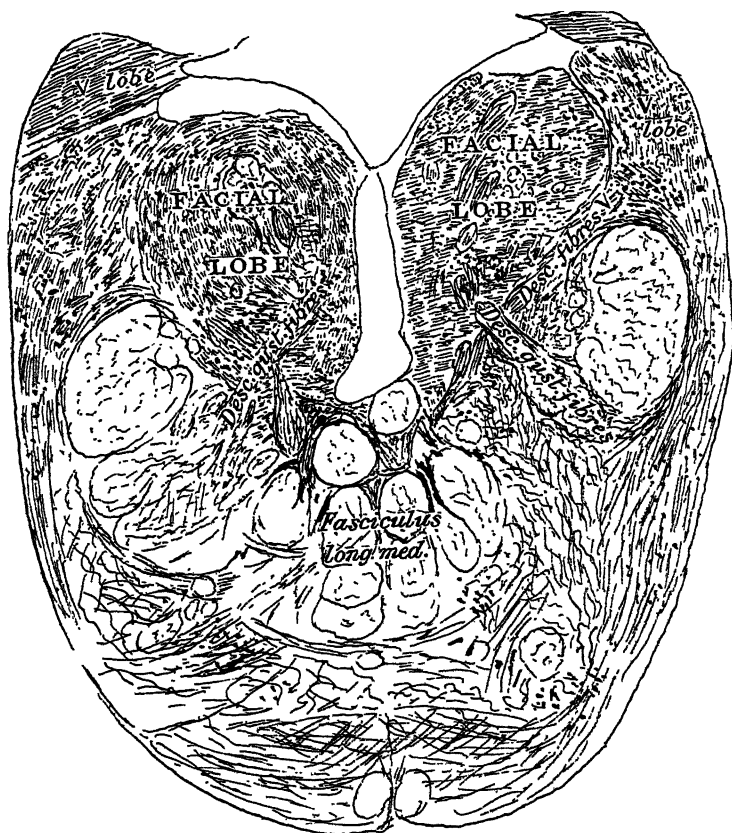
FIG. 12

acoustico-lateral lobes, but also the facial and glossopharyngeal lobes, and it is found that this method helps to confirm observations based on the relation of the various lobes to both nerve trunks and associated tracts.

PART II

A considerable variation in the hind-brain of Gadoids has been noted, and to give an idea of these changes we have prepared a series of drawings

of the dorsal aspect of the brain of six typical members of the cod family, fig. 18. The species with three dorsal fins and two anal are represented by the cod, the haddock, the whiting, and the pollack.

The species with two dorsal fins, the hinder long, the front one short, and one anal fin are represented by the ling, the hake, and the burbot, fig. 19. The species, in which there is no separate first dorsal fin, has a vibratile membrane in its position is represented by the rockling, fig. 19.
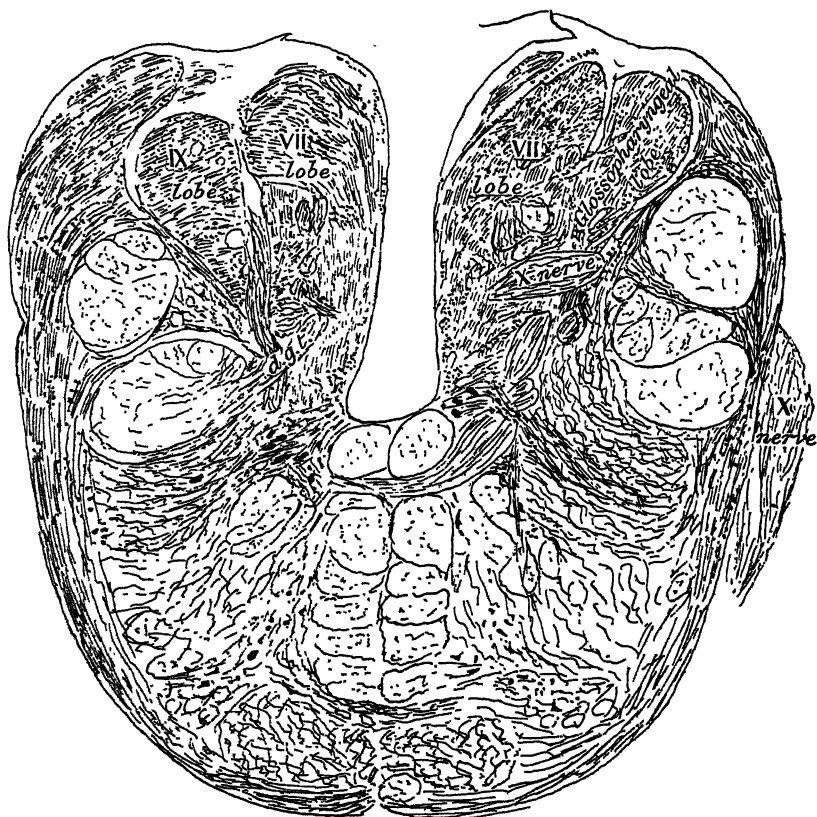
FIG. 13

Passing from above downwards column I, fig. 18, there will be noticed a gradual transition from a crustacean and molluscan diet to a purely fish diet. Passing again from above downwards, column III, it will be noted that the somatic sensory or V lobes at first small, gradually become very large and prominent.

Column IV shows an inverse transition in the size of the facial lobes. At the top of the list the facial lobes are large and passing downwards they become progressively smaller till the pollack and hake are reached.

The existence of barbels seems to be associated with two factors. The haddock and the cod each have a barbel which is no doubt used in their ground feeding habit. The whiting and pollack have no barbel, as their diet is nearly always fish. The same applies to the hake, but the ling and burbot, both night feeders, have a barbel, although the ling is a fish eater. The ling has also very large olfactory lobes.

We conclude, therefore, that a large somatic sensory lobe indicates a predacious habit, and a diet almost solely of other fish; while a large
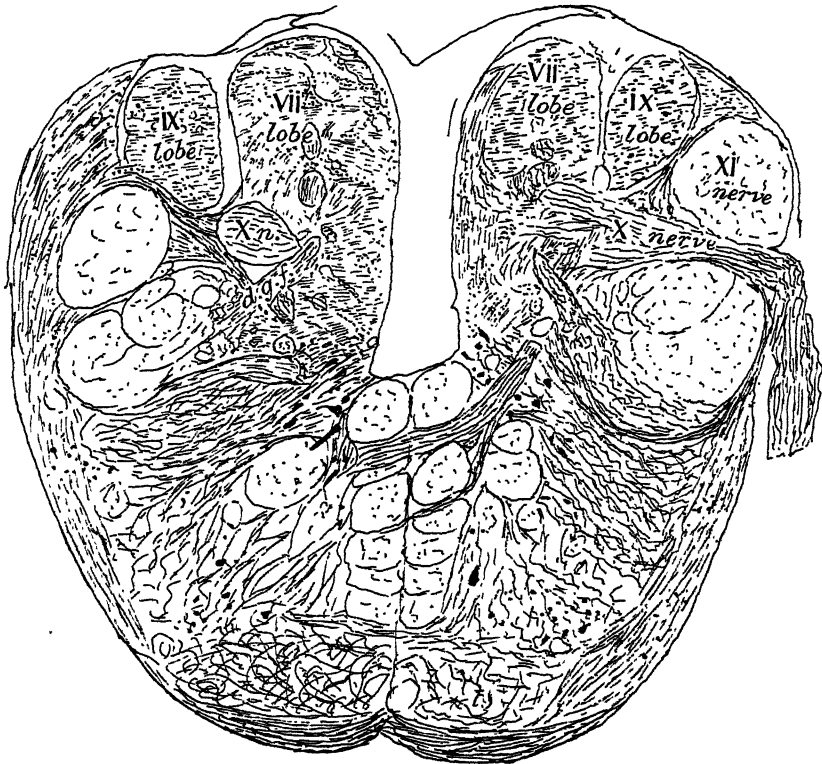
FIG. 14
In this fig. *xi* nerve should *read ix* nerve

facial lobe is found in bottom feeders in the diet of which mollusca, crustacea and worms are found predominant.

Outline Drawing of the Brain of Haddock (Gadus æglefinus), fig. 20*a*— Cerebellum removed; small somatic sensory lobes; large facial lobes; optic lobes, medium size.

Dentition—The upper jaw has a marginal area of small sessile teeth, 1·2 cm in length on each side, and 3 mm wide anteriorly. There is a

small area of dentition on the vomer. The lower jaw has similar teeth to the upper, of the same lateral extent but only 2 mm wide. The superior pharyngeal teeth are very prominent, 1·2 cm in antero-posterior diameter and 9 mm in lateral diameter. The longitudinal inferior dentate areas are 1·2 cm in length and 4 mm wide. There is a small barbel under the chin.

Diet—According to Cunningham (1896) the principal food in the Firth of Forth was found to be crustaceans and molluscs. The former were
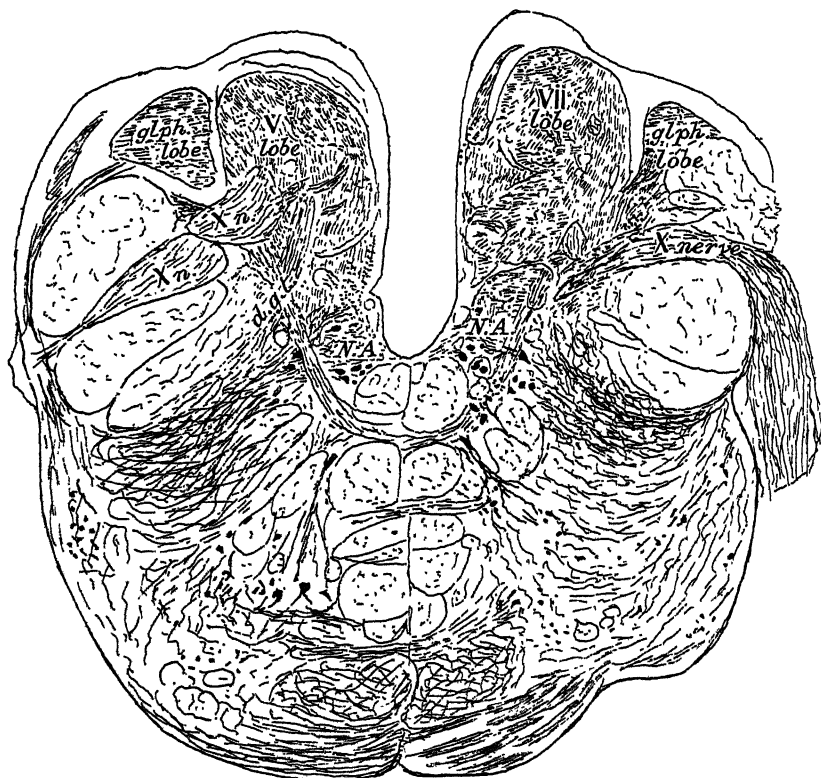
FIG. 15

found in 60% of the stomachs and the latter in 40%; echinoderms and worms were of nearly equal importance, 20% of the stomachs containing the former and 23% the latter. Fish were found in only 3%. Of the crustaceans, chiefly were found common shrimps, next hermit crabs, swimming crabs and sand hoppers.

Among molluscs common bivalves and the razor shell were most abundant; the whelk and other univalves, octopus and small cuttle fish also occurred.

a Whiting

b Roach
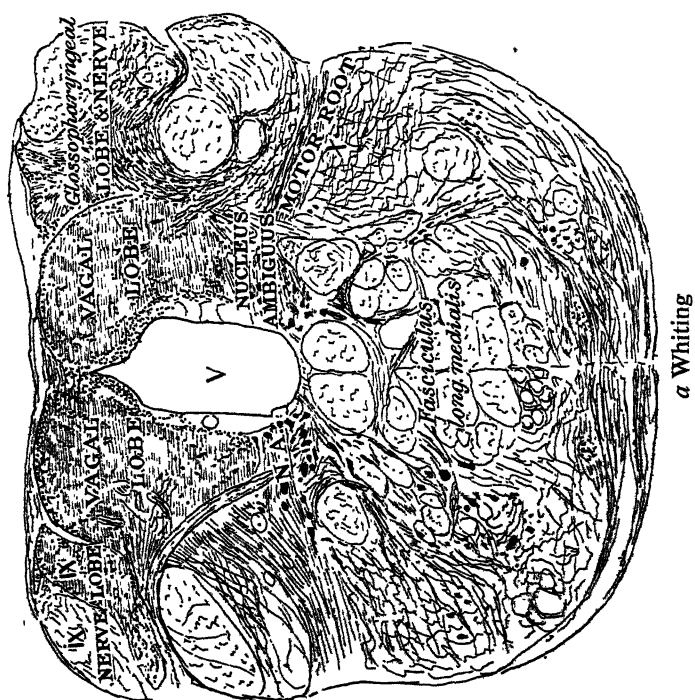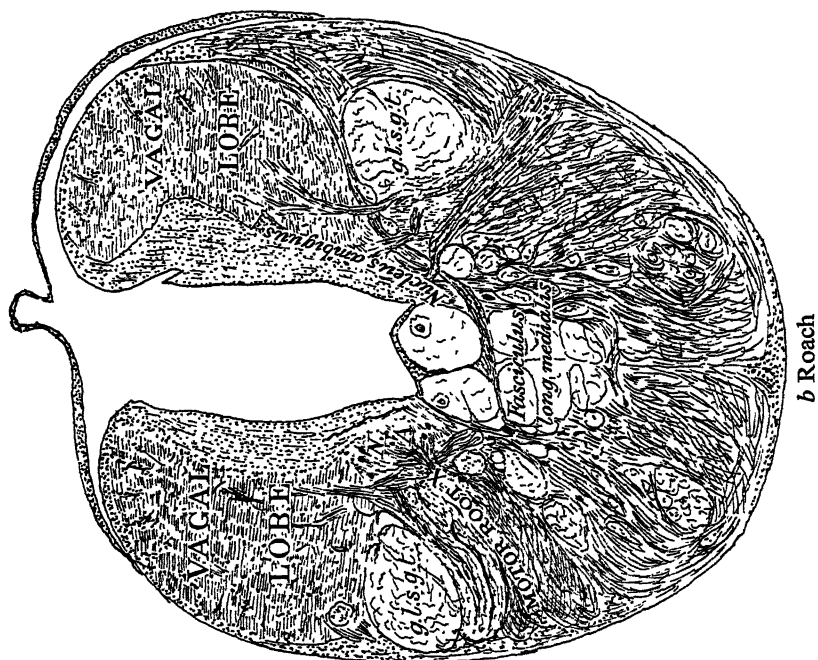
Fig. 16

Miss Thursby-Pelham notes herring spawn (whence the term applied to the fish at certain periods as " spawny haddocks ") large molluscs, as razor shell, polychaete worms, crustaceans and echinoderms.

To sum up we find large facial lobes associated with a dentition suited for crushing and a diet consisting mostly of crustaceans and molluscs.

Outline Drawing of the Brain of Cod (Gadus morrhua), fig. 20*b*—The long cerebellum has been turned forwards so as to expose the medulla oblongata.

The somatic sensory lobes are prominent and larger than those of the

FIG. 17

haddock. The facial lobes are well marked but definitely smaller than in the haddock, and they are overlapped by the posterior tailing of the somatic sensory lobes.

Dentition—Along the margin of the upper jaw are three rows of sharp teeth, moderate in length, the most anterior being the longest. They show a line of black pigment. They extend for 3·5 cm on each side, and their width in front is 6 mm. Within this row on the vomer is a V-shaped area with its apex projecting forwards with a rough tuberculated surface, the length of each arm is 1 cm and width 0·5 cm.

	I	II	III	IV	V
				LOBE	
	FOOD	HABITAT	LOBES	FACIAL	BARBEL

Haddock

a	Crustacea and mollusca echinoderms and worms	Feeds on the bottom	Optic lobes large somatic sensory *small*	Facial lobes *large*	Barbel present

Cod

b	Crustacea mollusca and fish	Leading demersal species and bottom feeder	Optic lobes large somatic sensory *medium*	Facial lobes *large* but smaller than haddock	Barbel present
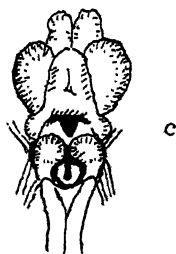
Whiting

c	Shrimps but mostly fish	Surface and day feeder	Optic lobes large somatic sensory *large*	Facial lobes *small*	No barbel

FIG. 18

I	II	III	IV	V
			LOBE	
FOOD	HABITAT	LOBES	FACIAL	BARBEL

Ling

I	II	III	IV	V
Fish almost entirely	Typical deep sea fish night feeder	Optic lobes small olfactory lobes *large* somatic sensory *large*	Facial lobes *small*	Barbel present
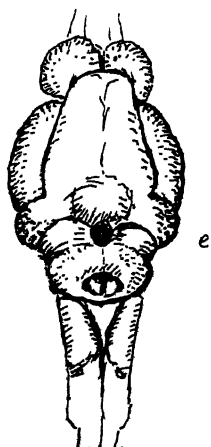
Pollack

I	II	III	IV	V
Fish rarely anything else when adult	Surface and day feeder prefers rocky ground	Optic lobes large somatic sensory *large*	Facial lobes *small*	No barbel

Hake

I	II	III	IV	V
Fish only	Deep water feeder	Optic lobes large acoustico- laterali large	Facial *minute*	No barbel

FIG. 18—continued
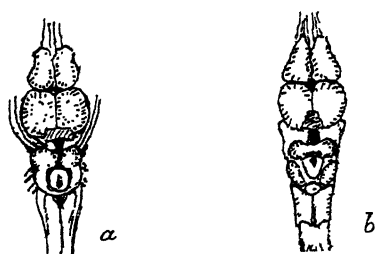
FIG. 19
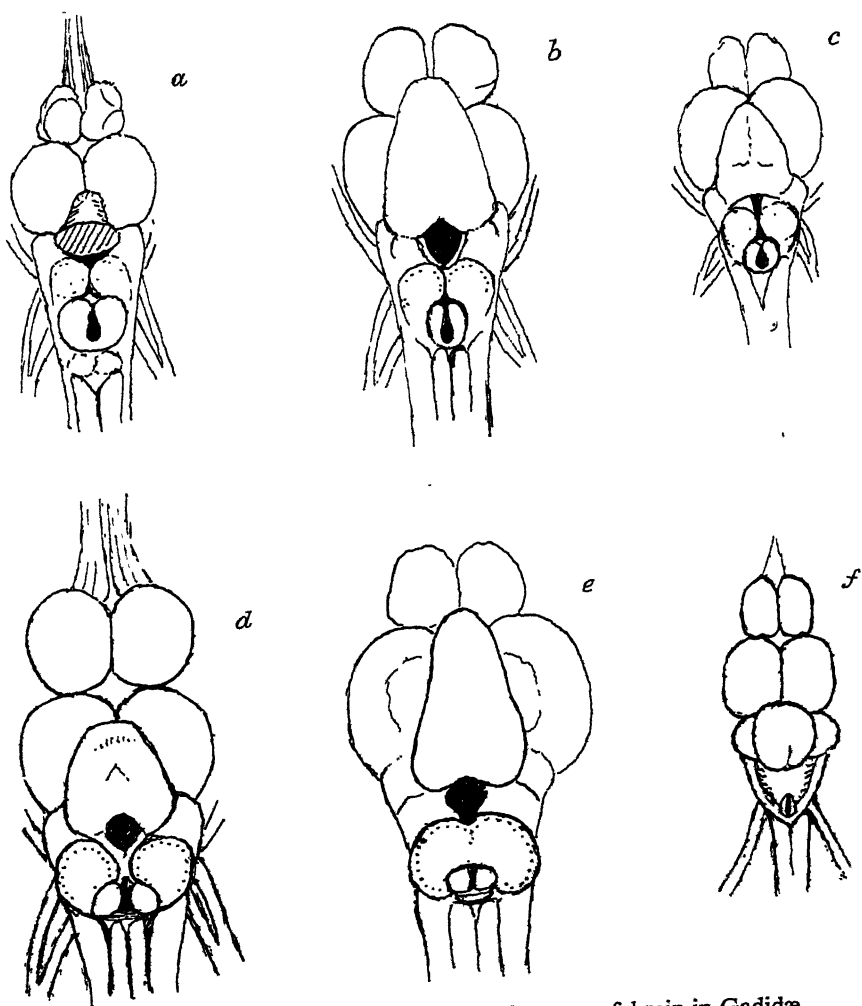
FIG. 20—Outline drawings to scale of six types of brain in Gadidæ

The lower jaw has similar teeth to the upper in three rows which extend 3·5 cm on each side. The pharyngeal teeth are separated by a soft tuberculated area triangular in shape, which extends on to the posterior margin of the palate. Projecting from the pharyngeal pad are sharp pigmented teeth. The pad is 1·7 cm in diameter. The inferior central ridges have a dentate area 2·5 cm by 7 cm. The gill arches are markedly tuberculated—there is a well-marked barbel.

Diet—The cod is a voracious feeder on other fish and also has a marked appetite for crustacea. Their diet is of whiting and herring, also hermit crabs, various large crustaceans such as the Norway Lobster, and molluscs and Aphrodite.

Outline Drawing of the Brain of Whiting (Gadus merlangus), fig. 20c— The cerebellum, as in the cod, has been turned forwards. The somatic sensory lobes are more globular and prominent than in the cod. The facial lobes are smaller and overlapped by the tailing of the sensory somatic lobes; compared with the haddock, the facial lobes are quite small. The optic lobes are large.

Dentition—There is a row of sharp pigmented teeth on the margin of the upper jaw; the longest may be 3 mm in length. They are separated by intervals of 2–3 mm. The lower jaw has similar teeth, but smaller.

There is a V-shaped area on the vomer as in the cod, but it is armed with small sharp teeth, each limb is 5 cm in length.

The superior pharyngeal pad as in the cod is armed with ˌsmall teeth; it is 7 mm in anterior posterior diameter and, 6 mm lateral diameter.

Diet—According to Miss Thursby-Pelham, the food of the whiting is mainly herring and whiting, and shrimps in large quantities.

Cunningham found that in the Firth of Forth their principal food was fish and crustacea, 65% of the former, 37% of the latter.

There is no barbel on the chin.

We note that there is a progressive diminution in the size of the facial lobe as we pass from haddock and cod to whiting, and an increase in the comparative size of the somatic sensory area. There is also a gradual change of dentition from the anterior crushing teeth of the haddock to the sharp teeth of the whiting, the vomerine teeth in the latter being sharp. The diet has also become mostly fish in the latter.

Outline Drawing of the Brain of Ling (Molva molva), fig. 20d—The cerebellum has been reflected forwards to expose the medulla oblongata. The primitive end-brain is large. The somatic sensory lobes are very

highly developed and send back prolongations which almost entirely envelop the small facial lobes.

Dentition—Within the margin of the upper jaw is a row of small sessile teeth extending backwards on each side for 4·5 cm. The dentate area
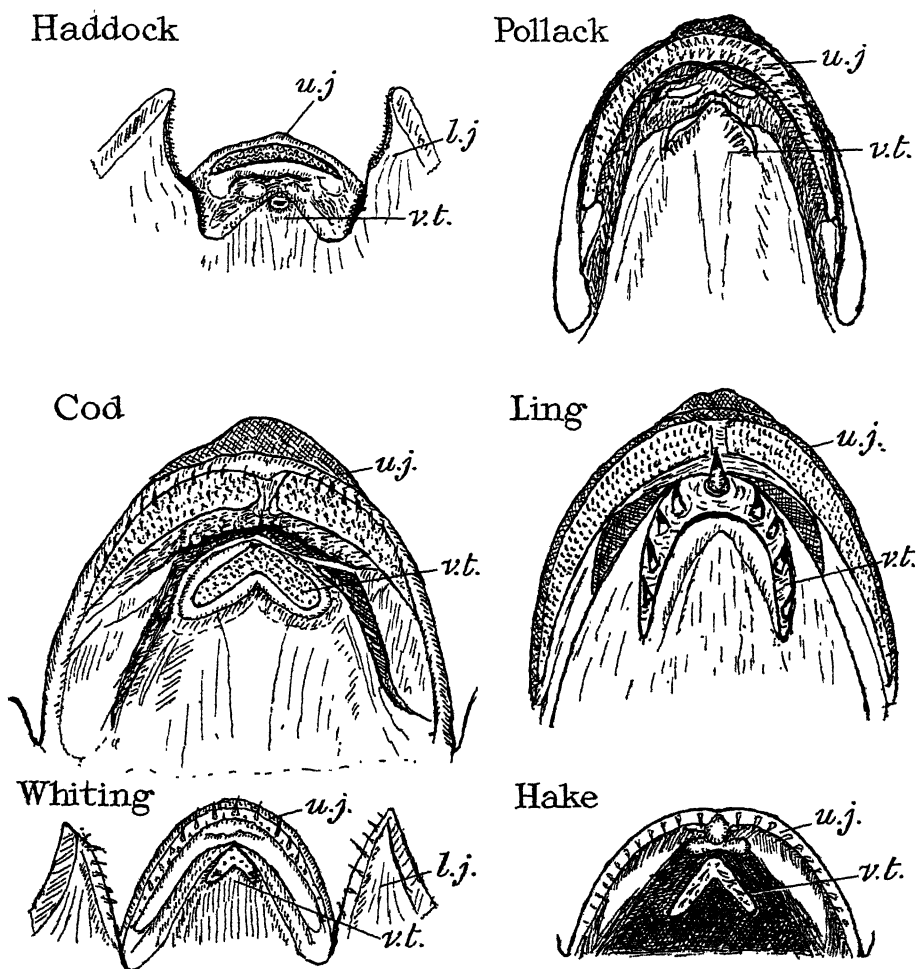
FIG. 21—Dentitions: *u.j.*, upper jaw; *l.j.*, lower jaw; *v.t.*, vomerine teeth. The lower jaw has been divided ventrally in haddock and whiting

is 7 mm in length anteriorly, and tapers gradually towards the angle of the jaw. Within this on the vomer are 6–7 prominent teeth widely separated, 5 mm in length. The lower jaw has smaller sharp teeth, eight in number on either side. Superiorly the area bearing the pharyngeal teeth is 2·5 cm in antero-posterior diameter and 1·5 cm laterally.

The inferior ridges are 3 cm long and 1 cm wide. There is a small lateral extension on each lateral margin of the superior pads which abut on the gill arches which are roughened. It has a well-marked barbel.

Diet—The ling is a voracious fish of prey and devours cod, whiting, mackerel, megrims, dabs, and haddock, and also crustacea and star-fish. All writers agree that it lives almost entirely on other fish. It is clear that the somatic sensory lobes are associated with its habits of chasing other fish and the small facials seem to point to crustaceans being a secondary article of diet. It is nocturnal in its habits.

Outline Drawing of the Brain of the Pollack (G. pollachius), fig. 20e— The cerebellum has been reflected forwards to expose the medulla oblongata. The optic lobes are large and the acoustic tubercles very prominent. The somatic sensory lobes are very marked and nearly hemispherical, with the posterior extremities embracing two very small facial lobes.

Dentition—Within the margin of the upper jaw are two rows of small sharp teeth with a central area in which there are four rows. The teeth extend for 3·5 cm on each side and towards the middle line the dentate area is 4 mm wide, but tapers as it passes backwards. Posterior to this row in the middle line on the vomer are two rows of small teeth extending for 6 mm and converging so as to form a V. On the margin of the lower jaw is a single row of small, sharp teeth.

The pharyngeal teeth are similar to those of the ling, but the area is smaller, being 1·5 cm in antero-posterior diameter, and 1 cm lateral diameter. Inferiorly the median longitudinal ridges are narrow and 2·8 cm in length. The gill arches are tuberculated with fine points. It has no barbel.

Diet—This fish is a voracious feeder on herring, sprats, pilchards, and mackerel. It is purely a fish eater. The sharp teeth, large eyes and big sensory somatic area and small facial all point to its feeding by sight and touch on fish inhabiting the upper waters of the sea. The small facials are in marked contrast to the large lobes of the molluscan and crustacean feeder like the haddock.

Outline Drawing of the Brain of the Hake (Merluccius vulgaris), fig. 20f— The appearance of the medulla oblongata viewed from above is strikingly different from that of those Gadidæ we have already described. The cerebellum does not project over the rhomboid fossa and is ovoid and bounded by very prominent acoustic tubercles, from which narrow ridges pass back enclosing a wide rhomboid fossa and terminating at the sides

of minute facial lobes. The pattern is somewhat similar to the medulla of the burbot.

Dentition—The margins of the upper and lower jaws are armed with sharp pointed teeth slightly curved inwards and movable towards the inner side. They are set widely apart so that there are approximately 15 fully-grown teeth on each side of the jaw. The longest teeth are 3 mm long. On the vomer is a V-shaped area, the apex looking forwards, each arm is 1·1 cm long and the base is 1·1 cm wide. The dentate area bears a number of small pointed teeth similar to those on the jaws.

Diet—The hake is a very voracious feeder, living on other fish such as herring, pilchards, and mackerel. The absence of crustacea and mollusca in their diet is associated with the presence of the small facial lobe, which is minute in size as seen by the naked eye.

It is a curious fact that the medulla is very similar to that of the burbot, also purely a fish eater.
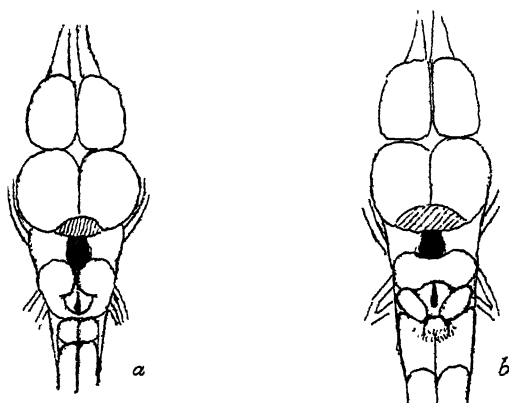
FIG. 22

Outline Drawing of the Brain of Burbot (Lota vulgaris), fig. 22a—The cerebellum has been cut off at its base. The primitive end-brain is large, almost as long in its antero-posterior diameter as the optic lobe. The acoustico-lateral areas are large. The somatic sensory lobes are large, prominent, and almost hemispherical. They are prolonged posteriorly by two extensions which surround the small facial lobes which are deeply seated.

Dentition—" The mouth is wide with bands of small pointed teeth in the jaws, and a crescentic band of similar teeth on the vomer " (Tate Regan, 1911). The teeth within the margin of the upper jaw are very small and they approximate closely the band on the vomer. The vomerine

teeth are longer, recurved and pigmented as in the whiting. The band on the lower jaw is narrower than on the upper, but the teeth are longer and recurved. The pharyngeal pads are smaller and not prominent. It has a barbel depending from the lower jaw, whilst the edge of each anterior nostril is produced into a small barbel.

Diet—According to Tate Regan (1911) it is a " gluttonous fish and eats great quantities of the eggs of other species. It is especially destructive to other fish. In the day time it pounces on any small fish which comes near its lurking place, whilst at night it goes in active pursuit of prey."

The Brain of the Three-bearded Rockling (Motella vulgaris), fig. 22*b*— The cerebellum has been removed. The primitive end-brain as in the burbot is large, approaching the optic lobes in length, but narrower and tending to taper anteriorly into the large olfactory stalks. The optic lobes are of medium size compared with other Gadidæ. The acoustico-lateral areas are large. The somatic sensory lobes have the appearance of a dumb-bell transversely situated, and instead of tailing off posteriorly as in ling or burbot, there are two obliquely lying ovoid protuberances on either side. The vagal lobes are large and a prominence projects from their anterior ends between the lateral ovoid lobes.

Dentition—There is a row of small teeth within the margin of both upper and lower jaws and on the vomer a triangular area covered with small teeth. This fish has three barbels, the lower jaw bearing one centrally situated and, the upper jaw two, with the nasal aperture at their base. But a peculiar organ is developed from the dorsal fin. To quote Couch (1866) " the organ often represented as the first dorsal fin—lies in a chink from which it projects when the fish is in the water. It is formed of a membrane from the edge of which rises a thickly placed row of threads, the foremost of which is the stoutest and the most prominent. This organ is in continued and rapid motion. It is well furnished with nerves which render it acutely sensible to impressions." Microscopically it is found to be well provided with taste buds. " This dorsal organ is supplied by a special nerve which reaches it directly from the brain, a branch of this nerve also goes to the pectoral and ventral fins which are thus endued with particular powers of sensation " (they are also provided with taste buds) " in addition to those of action, the last-named faculty being controlled by branches of the intercostal nerves."

These observations are the result of the examination of the three bearded rockling and are of interest as showing the accurate anatomical observations of older authors.

The Brain of the Five-bearded Rockling, fig. 23—The specimen from which the figure has been drawn was specially hardened as for cutting serial sections. By this method more detail of the formation of the various lobes has been obtained. The acoustico-lateral areas are very marked. The somatic sensory lobes are slightly prolonged caudally, and have been widely separated by the very large facial lobes which appear more rounded in outline than the oval shaped prominences shown in fig. 22, *b*. The vagal lobes are very prominent dorsally.

Fig. 24 shows six serial sections from one of the same batch of five-bearded rocklings from which the drawing, fig. 23 was made.

Fig. 24, *a*, shows the facial nerve entering transversely.

Fig. 24, *b*, shows the prominent somatic sensory lobes and the commencement of the facial lobes. Figs. 24, *c* and *d*, show the descending fibres passing from the facial lobes into the great longitudinal secondary gustatory tracts.

Fig. 24, *e*, shows the very hypertrophied facial lobes and the commencement of the vagal lobes, and fig. 24, *b*, the vagal lobes increasing in size and the large cells of the nucleus ambiguus.

The distinctive pattern of the medulla oblongata as seen under low power and in the serial sections points to the conclusion that the very large facial lobes are due to the presence of the peculiar area in the site of the dorsal fin which is supplied with taste buds, as described above.

FIG. 23—Five-bearded rockling. × 10. *clm.*, cerebellum divided at base; *s.s.l.*, somatic sensory lobe; *f.l.*, facial lobe; *a.t.*, acoustic tubercle; *vag.l.*, vagal lobe.

Diet—Their food is for the most part the smaller crustaceans and worms. They feed also on small fish and hunt only at night.

The medulla oblongata, as already shown, has a different pattern from that of other Gadidæ. Associated with the vibratile membrane and groove in the site of the first dorsal fin we find that the facial lobes are hypertrophied.

The rocklings, therefore, form a group by themselves, not purely crustacean and molluscan in diet as the haddock, but like the burbot in their nocturnal habits. The large primitive end-brain points to well-developed olfactory function, and the optic lobes being poorly developed point to their night feeding habits.

The study of a complete series of sections seems to justify the conclusion that there is a bilateral facial lobe in whiting; with the same nerve connections as in the Cyprinoids, a facial nerve entering transversely and then turning sharply backwards to enter a posteriorly situated facial lobe, and secondly, descending gustatory fibres passing into the great longitudinal gustatory tract on either side.
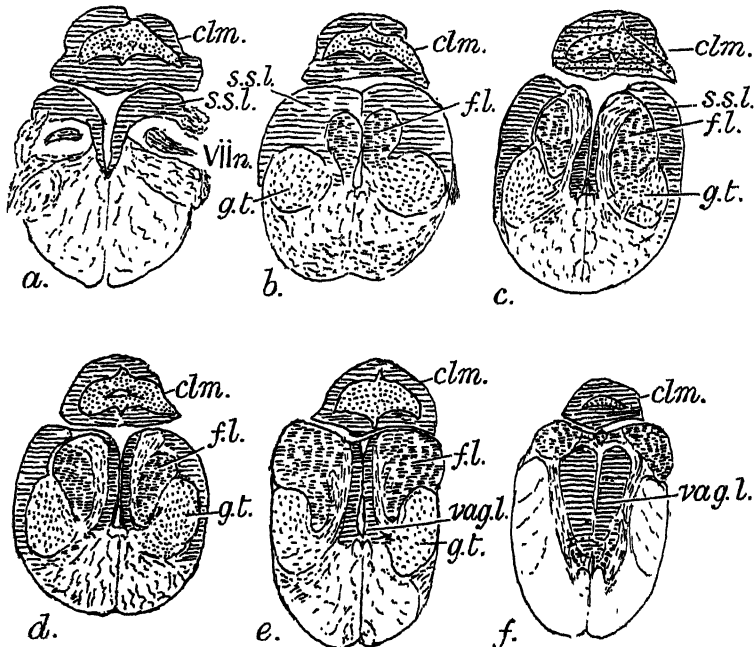
FIG. 24—Medulla oblongata of rockling. *clm.*, cerebellum; *s.s.l.*, somatic sensory lobe; *f.l.*, facial lobe; *vag.l.*, vagal lobe; *g.t.*, gustatory tract. (*a*) Section at level of entrance VII nerve; (*b*) across VII lobe; (*e*) across hypertrophied portion of VII lobe; (*f*) across vagal lobe.

We have been able to separate in the posterior crura an acousticolateral portion with a motor area, from a somatic sensory area, which is composed of a V nerve element. This is connected with another definite motor area. The vagus enters in its usual position and the nucleus ambiguus is present in the same site as in Cyprinoids. In Gadidæ the somatic sensory area is highly developed, while the vagal lobes are small compared with those in the Cyprinoids. This feature is particularly marked in the predacious members of the the cod family. The facial lobes are well developed only in those fish which feed largely on crustaceans, molluscs, and worms.

Associated with these habits of feeding, we also find a progressive change in the dentition from teeth adapted to crushing shellfish, as in haddock, to the sharp teeth of the fish-eating members of the family, as the ling and hake.

SUMMARY

The type of medulla oblongata in the Gadoids has been described and compared with the type found in Cyprinoids.

The variation in the pattern of the medulla in different species has been shown to be related to their habits of feeding, which are associated with changes of type in their dentition.

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Experiments on the Intracerebral Implantation of Embryo Tissues in Rats

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[PLATES 22–23]

INTRODUCTION

The experimental implantation of embryo tissues into adult or growing animals of the same species has been practised both by embryologists and by pathologists—by the former in the study of the capacity of the implanted tissues for independent growth and differentiation, and by the latter usually in the hope (which has not been realized) of shedding light on the genesis of tumours and especially of the teratomata. Fishes, amphibians, birds, and mammals have been used in these studies; and the more important of the easily accessible accounts include those of Zahn (1884), Féré (1895–1903), Birch-Hirschfeld and Garten (1899), Traina (1902), Nichol (1905), Del Conte (1907), Askanazy (1907, 1926), Petrow (1908), Tiesenhausen (1909), Shattock and co-workers (1910), Freund (1911), Dietrich (1912), Reinke (1913), Weber (1920–23), Bosaeus (1926) (who gives a complete and very useful summary of the literature up to 1926), Pichler (1933), and Nicholas (1934).

In general, the results which have been obtained may be summarized briefly as follow. Embryos or parts of embryos, implanted whole or minced or pulped into adult animals of like species, may suffer any of three fates: (i) the tissues may be rapidly and completely absorbed, or (ii) they may survive, grow, and differentiate for some days or weeks, and then suffer resorption, or (iii) they may grow to considerable masses of mixed differentiated tissues which may persist for long periods, even for the lifetime of the host animal. The results vary with the species of animal, the age and degree of subdivision of the embryo tissue introduced, the site of implantation, and probably the age, health, and other personal peculiarities of the host animal. Although some workers have claimed to observe the development of tumours from the implanted tissues, none of the instances of this alleged transformation can be accepted as proved.

The present study began in an attempt to repeat the observations of Askanazy and others who found that white rats appeared to be particularly suitable for the production of " teratoid " masses from subcutaneous or intraperitoneal implants of embryo tissues. My results with sub-cutaneous inguinal implantation in white rats were, however, very dis-appointing. Of 25 animals treated with tissues from embryos varying from 8 to 35 mm long and killed at periods from 6 to 13 weeks later, only 2 animals presented masses of any interest; of the remaining 23, 3 exhibited small residual nodules of cartilage only, while in 20 the im-planted tissues had been completely absorbed. It was indeed remarkable how a whole head or trunk of a nearly full-term fœtus would vanish in a few weeks, leaving not a trace even of the bones. The two rats in which considerable masses were found had each had bilateral implants con-sisting of whole heads of 1·5 cm embryos. Killed in 13 weeks, one animal presented masses in each groin, the larger 1·2 cm in diameter, containing nodules of bone and cartilage, epidermal cysts, mucous cysts, striated muscle-fibres, and some central nervous and ganglionic tissue with nerve-cells; while in the second rat the masses were smaller, and contained bone, cartilage, mucous cysts, striated muscle-fibres, a tooth, and a nodule of thyroid tissue.

Equally fickle results followed the intratesticular implantation of small fragments of embryo tissue; in nearly all instances the tissues failed to survive and had disappeared when the testes were examined micro-scopically 6 to 8 weeks later.

Intracerebral implantation was then tried; and, contrary to the findings of Del Conte and Tiesenhausen, it was found possible by this method to obtain large masses of growing tissues in a high proportion of the treated animals. So striking have the results been, that it was thought desirable to record details of the technique and results of these experiments.

TECHNIQUE

An identical technique was used in all experiments. Pregnant white rats were killed by chloroform and the embryos removed at once to warm normal saline. The parts of the embryos to be used were removed with sharp small scissors, washed in salt solution, freed of excess fluid by blotting on filter paper, placed in a watch-glass and cut up into frag-ments small enough to enter easily the bore of a lumbar puncture needle. The approximate average diameter of the fragments, estimated under the microscope, was about 200 μ. For each implantation the lumbar punc-ture needle was loaded by applying its bevelled point to the moist mass

of minced tissue and withdrawing the stilette about 1 cm, thus sucking a portion of the mince into the end of the needle. The average amount of tissue with which the needle was loaded for each injection was roughly between 2 and 5 mg. During these operations no special care was taken to keep the minced tissues warm, but sterility was aimed at throughout. From each batch of embryos, at least one was saved for microscopical section to determine the degree of differentiation of the tissues at the time of implantation.

The host animals were young, healthy, white rats of ages ranging from 2 weeks to 4 months. No close blood relationship existed between the embryos used and the host rats; both were taken at random from the general stock. Light ether anæsthesia was used. The hair at the area to be punctured or incised was clipped short and the skin swabbed with spirit. In the four-month animals, a short incision was made through the scalp and a small hole was bored through the skull to admit the lumbar puncture needle; but in younger animals no incision or trephining was needed, the needle being introduced through the skin of the neck in the mid-dorsal line and advanced to the skull which was then gently pierced in an oblique direction. In most instances the skull was punctured through the right parietal bone or through the interparietal suture; the needle was advanced 2 or 3 mm, and the stilette thrust home to expel the contents, usually into the right cerebral hemisphere. In a few animals withdrawal of the needle was followed by some venous bleeding from the puncture in the skull, but this soon ceased. Of the 45 rats treated, only one (in which the brain-stem was pierced) died as the direct result of the operation; and all except No. 15 showed no signs of any disturbance of brain functions. In several of the groups of animals control implants, similar in kind and quantity to those in the brains, were made also into the subcutaneous tissues of the neck.

The rats were killed at periods ranging from 4 to 13 weeks later, and the neck, scalp, skull, and brain carefully examined. In animals in which an obvious intracranial mass was visible through the exposed skull (and this applied to nearly one-half of them), the skull and its contents were fixed entire; but in others the skull was opened and the brain removed before formalin-fixation. All specimens were decalcified, since most of them contained bone or teeth. Paraffin sections were made through the whole brains in frontal planes; through parts of the brains containing masses of embryo tissue, representative sections were taken at intervals of half a millimetre or less. Sections of any masses found in the neck, scalp, or skull were also prepared. The tissues from each host-rat were kept separate. The stains used were hæmalum followed

by eosin, picric acid, or Van Gieson's stain, and Heidenhain's iron-hæmatoxylin followed by Van Gieson.

RESULTS OF THE EXPERIMENTS

In the following account the experiments are arranged in ascending order of the age of the embryo tissues used. Only the briefest possible statement of the tissues identified in the growing implants is given; and description of the degenerative and phagocytic phenomena seen in many of the specimens is reserved for the discussion. The measurements given of the masses of tissue represent their longest, usually their antero-posterior, diameters. The illustrations amplify the descriptions.

Experiment I   (Rats 1 to 3)

Embryo tissues used were from very early gestation, the uteri presenting solid decidual swellings 4 mm in diameter embedded in which were blastocysts 1·2 mm in main diameter. Four of these decidual masses were peeled free of uterine wall and minced up for injection.

Hosts—Three 2-week rats received intracranial and cervical implants.

Results—All killed in 6 weeks; no residual tissues found.

Experiment II   (Rats 4 to 8)

Embryo tissues used—Heads of 1·5 cm embryos.

Hosts—Five 3-week rats received intracranial implants.

Results—All killed in 13 weeks. *No.* 4 had a mass 8 mm in diameter in the left hemisphere, containing cartilage, bone with hæmopoietic marrow, adipose tissue, striated muscle-fibres, epidermal cysts with hairs and sebaceous and sweat glands, and one well-formed incisor tooth 4 mm long. *No.* 5 had a mass 5 mm in diameter in the right hemisphere, containing bone and squamous epithelial cysts. *No.* 6 had a mass 10 mm in diameter in the left hemisphere, containing bone, adipose tissue, striated muscle-fibres, squamous epithelial cysts, mucous cysts, and serous salivary gland tissue. A small nodule in the scalp contained cartilage, bone, and epidermal cysts. *No.* 7 had a mass 6 mm in diameter lying between and encroaching on both hemispheres, containing bone with hæmopoietic marrow, adipose tissue, striated muscle-fibres, mucous cysts, and serous salivary gland tissue. *No.* 8 negative.

Experiment III   (Rats 9 to 14)

Embryo tissues used—Lower jaws and tongues of 1·6 cm embryos.

Hosts—Six 5-week rats received intracranial and cervical implants.

Results—*No.* 9, killed in 4 weeks, had a huge mass 12 mm in diameter involving the right lobe of the cerebellum, the posterior half of the right hemisphere and the mesencephalon; containing bone with hæmopoietic marrow, adipose tissue, striated muscle-fibres, epidermal cysts, and serous salivary gland tissue. A mass 10 mm in main diameter in the neck contained cartilage, bone, adipose tissue, epidermal cysts, mucous cysts, serous salivary gland tissue, and part of a tooth. *No.* 10, killed in 7 weeks (opisthotonic convulsions during anæsthesia), had a large mass 11 mm in diameter in the right hemisphere with marked crowding of the brain stem and left hemisphere to the left; containing bone, adipose tissue, striated muscle-fibres, epidermal cysts, mucous cysts, one cyst lined partly by skin and partly by pseudo-stratified ciliated epithelium of respiratory type, and one tooth. The neck contained one mass 10 mm in main diameter and several smaller masses, containing bone, cartilage, epidermal cysts, mucous cysts, and one well-formed molar tooth. (Because of the unusually vigorous' growth of the tissues in this case, it was thought wise to attempt secondary transplantation to other animals. Six 4-week rats were given intracranial and cervical injections of minced tissue from one of the large masses in the neck of *No.* 10; they were killed 4 weeks later; minute degenerating foci of cartilage, bone, or squamous epithelial cysts were present in the necks of three and in the brains of four of the animals; but these were clearly only non-proliferating residues of the injected tissues.) *No.* 11, killed in 7 weeks, had a mass 5 mm in diameter in the right hemisphere; containing cartilage, squamous epithelial cysts, and mucous cysts. Several smaller masses in the neck contained only squamous epithelial cysts. *No.* 12, killed in 9 weeks, had a mass 9 mm in diameter in the right hemisphere, occupying the posterior part of the ventricle, the anterior part of which presented marked hydrocephalic distension; the mass contained bone, striated muscle-fibres, and epidermal cysts. Several masses in the neck, up to 8 mm in diameter and of total weight 0·2 gm, contained cartilage and epidermal cysts. *No.* 13, killed in 9 weeks, had a mass 8 mm in diameter in the right hemisphere; containing bone with hæmopoietic marrow, adipose tissue; striated muscle-fibres, epidermal cysts, mucous cysts, and one well-formed tooth. A mass 8 mm in diameter in the neck contained bone, epidermal cysts, and mucous cysts. *No.* 14, killed in 9 weeks, had a mass 6 mm in diameter in the right hemisphere; containing epidermal cysts. Several small nodules in the neck contained epidermal cysts and part of a tooth.

Experiment IV   (Rats 15 to 20)

Embryo tissues used—Upper jaws, nasal cavities, and eyes of 1·6 cm embryos.

Hosts—Six 8-week rats received intracranial and cervical implants.

Results—*No.* 15, exhibited from the time of operation severe ataxia and forced rotation to the right side, and died in convulsions in 3 weeks. A mass 7 mm in diameter occupied the right half of the brain-stem and right lobe of the cerebellum; containing cartilage, bone, epidermal cysts, and much inflamed connective tissue infiltrated by lymphocytes and polymorph leucocytes (? infection of the implant).   Nil in the neck. *No.* 16, killed in 7 weeks, had some small nodules in the leptomeninges only, containing cartilage and squamous epithelial cysts.   Nil in the neck.   *No.* 17, killed in 8 weeks, had a mass 6 mm in diameter in the right hemisphere; containing bone, cartilage, smooth muscle-fibres, epidermal cysts, mucous cysts, and a tooth.   The neck contained one tiny cartilaginous nodule only.   *No.* 18, killed in 8 weeks, had some small nodules in the leptomeninges; containing cartilage, bone, and degenerating foci of keratin.   The neck contained a tiny nodule of cartilage.   *No.* 19, killed in 8 weeks, had a mass of cartilage and squamous epithelial cysts in the meninges.   Nil in the neck.   *No.* 20, killed in 8 weeks, had an incomplete tooth and some spicules of bone in the meninges. The neck contained some small cartilage nodules.

Experiment V   (Rats 21 to 25)

Embryo tissues used—Tongues of 2 cm embryos.

Hosts—Five 2-week rats received intracranial implants.

Results—*No.* 21 died in 4 weeks; a mass 8 mm in diameter in the right hemisphere, with hydrocephalic distension of the anterior part of the ventricle; containing adipose tissue, many striated muscle-fibres, mucous cysts, and squamous epithelial cysts devoid of hairs and cutaneous glands and with a curious ridging of their lining and of their·contained strata of keratin.   *No.* 22, killed in 4 weeks, negative.   *No.* 23, killed in 8 weeks, had a mass 7 mm in diameter involving both hemispheres; containing much striated muscle, and squamous epithelial cysts like those of *No.* 21.   *No.* 24, killed in 8 weeks, had a mass 6 mm in diameter in the right hemisphere, with hydrocephalic distension of the unoccupied parts of the ventricle; containing much striated muscle, and squamous epithelial cysts like those of *No.* 21.   *No.* 25, killed in 8 weeks, had a dis-

tended skull, and a huge mass 12 mm in diameter in the right hemisphere, crowding the rest of the brain to the left; the mass contained skeletal muscle and squamous epithelial cysts devoid of hairs and skin-glands.

Experiment VI   (Rats 26 to 34)

Embryo tissues used—Upper and lower jaws (minus tongues) of 2-cm embryos.

Hosts—Nine 4-month rats received intracranial implants.

Results—*No.* 26, killed in 4 weeks, had a mass 6 mm in diameter in the right hemisphere; containing cartilage, bone, striated muscle-fibres, epidermal cysts, and a tooth. *No.* 27, killed in 6 weeks, had a mass 8 mm in diameter in the right hemisphere; containing cartilage, bone, epidermal cysts, mucous cysts, and a tooth. *No.* 28, killed in 6 weeks, had a mass 6 mm in diameter in the right hemisphere; containing cartilage, bone, squamous epithelial cysts, mucous cysts, and a tooth. *No.* 29, killed in 6 weeks, had a mass 4 mm in diameter in the wall of the right ventricle; containing cartilage, bone, and epidermal cysts. *No.* 30, killed in 9 weeks, had a mass 9 mm in diameter in the right hemisphere; containing cartilage, bone, striated muscle-fibres, epidermal cysts, mucous cysts, and serous salivary gland tissue. *No.* 31, killed in 9 weeks, had a mass 11 mm in diameter in the right hemisphere; containing cartilage, bone with hæmopoietic marrow, epidermal cysts, mucous cysts, and a tooth. *No.* 32, killed in 9 weeks, had a few small nodules of cartilage in the meninges only. *No.* 33, killed in 9 weeks, had a mass 4 mm in diameter in the wall of the left ventricle; containing cartilage, epidermal cysts, and a molar tooth. *No.* 34, killed in 9 weeks, had a mass 8 mm in diameter in the right hemisphere; containing cartilage, bone, striated muscle-fibres, epidermal cysts, and mucous cysts.

Experiment VII   (Rats 35 to 45)

Embryo tissues used—Head of 3·5 cm (*i.e.*, nearly full-term) fœtus, discarding brain.

Hosts—Eleven 4-week rats received intracranial and cervical implants.

Results—*No.* 35, killed in 7 weeks, had a mass 4 mm in diameter in the right hemisphere; containing cartilage, adipose tissue, and epidermal cysts; and a mass of similar size and structure in the neck. *No.* 36, killed in 7 weeks, had a dilated right ventricle into which projected small epidermal nodules and hairs. Nil in the neck. *No.* 37, killed in 7 weeks, had a small nodule in the left hemisphere; containing bone and

a squamous epithelial cyst. Nil in the neck. *No.* 38, killed in 9 weeks, had a mass 5 mm in diameter in the right hemisphere; containing cartilage, bone with hæmopoietic marrow, and squamous epithelial cysts. Nil in the neck. *No.* 39, killed in 9 weeks, had a small nodule of bone with hæmopoietic marrow in the right hemisphere. The neck contained a small nodule consisting of bone, epidermal cysts, and serous salivary gland tissue. *No.* 40, killed in 9 weeks, negative. *No.* 41, died (of enteritis) in 9 weeks; and had an incomplete tooth in the wall of the right ventricle. Nil in the neck. *No.* 42, killed in 11 weeks, had a small mass of bone and epidermal cysts in the right hemisphere. The neck contained a small nodule of cartilage. *No.* 43, killed in 11 weeks, had a small focus of squamous epithelial cysts in the neck, and nil in the brain. *No.* 44, killed in 11 weeks, had some small nodules of degenerating cartilage and squamous epithelium in the choroid plexus of the left ventricle, and nil in the neck. *No.* 45, killed in 11 weeks, had some small bony foci in the choroid plexus and wall of the left ventricle, and nil in the neck.

DISCUSSION

A—The Degree of Success of the Method—In these experiments, intracranial implantation has proved more successful than subcutaneous implantation. Of the entire series of 45 rats, successful intracranial implantation was obtained in 38 (in the brain in 33, in the leptomeninges in 5). Of 26 rats which received both intracranial and cervical implants, the former gave positive results in 21 and the latter in 13; and, in general, successful intracranial implants yielded masses of tissue which were larger and contained a greater variety of tissues than successful cervical implants. However, as shown by experiment III, implants which grow vigorously in the brain may grow very well in the cervical tissues also. In view of my disappointing results in the separate series of rats with inguinal implants, I suspect that the neck may be a more favourable site than the groin; but as I have not introduced implants in both sites in any animals, I cannot affirm that this is so.

The meninges and the choroid plexus appeared to be less favourable than brain tissue for the survival and growth of implants (see rats 16, 18, 19, 20, 32, 44, and 45). In these situations, the nodules of surviving tissue were small, and often undergoing degeneration and phagocytic absorption.

The age of embryo tissues used decidedly influenced the results. Tissues from half-grown embryos of 1·5 or 2 cm (experiments II to VI) gave much better results than those of the nearly full-term fœtus (experiment

VII); while very early embryos minced up along with the surrounding decidua failed to survive (experiment I). These results accord with those of Askanazy and others. My experiments are, of course, too few to decide the optimum age of the embryo tissues for implantation.

B—The Tissues Obtained in Successful Implants—In order to minimize difficulties in the identification of the tissues in established implants, I used only embryo heads or parts of heads in these experiments; and all of the components in the growing implants corresponded to various normal cranial structures. The most abundant were cartilage and bone, epidermis, mucosal epithelia, teeth, and skeletal muscle; while serous salivary gland tissue, adipose tissue, and smooth muscle-fibres were seen less frequently. No surviving central nervous or ocular structures were found. Some at least of the blood vessels, connective tissues, and collections of lymphocytes seen in the implants were undoubtedly derived from the implanted tissues, but, of course, these could not be distinguished with certainty from the tissues of the host.

Certain features of individual tissues deserve comment. As to *bone*, active ossification in cartilage was evident in several specimens, while in other cases the bone was clearly membrane-bone. Some bony masses, fig. 1, Plate 22, contained richly cellular hæmopoietic marrow with sinusoidal blood-channels and sometimes megakaryocytes. *Epidermal cysts* were readily identified by their hairs and sebaceous and sweat glands which were often plentiful and fully developed. The contents of these cysts consisted of sebum and keratin. Other keratin-filled stratified epithelial cysts, devoid of hairs and cutaneous glands, doubtless represented *oral or pharyngeal epithelium*. In experiment V, the ridging in the lingual epithelial cysts and in the layers of keratin within them probably corresponded to the filiform and other papillæ of the normal tongue, fig. 2, Plate 22. *Mucous cysts* were of several varieties, some lined by simple goblet-celled epithelium, others by pseudo-stratified epithelium with or without cilia. It was usually impossible to decide from what particular normal epithelium these cysts had arisen; probably nasal, salivary, and respiratory epithelia all participated. *Teeth* were sometimes so well formed that they could be identified as incisors or molars, figs. 3 and 4, Plate 22; in other cases they were abnormal in shape or incomplete. In one specimen the base of a tooth abutted directly against the cerebral tissue of the host, vessels from which had grown into the dental pulp, fig. 5, Plate 23. Incomplete teeth were of interest in that they resembled cut-off pieces of otherwise well-developed teeth; evidently a fragment of a tooth-germ in a half-grown embryo has no power of regulation to

form a complete tooth. The structural details of the teeth were often beautifully clear, fig. 6, Plate 23. *Serous salivary gland tissue*, both acini and ducts, in several specimens appeared quite normal in structure. Presumably any secretions formed by these glands were discharged into neighbouring cysts. *Striated muscle-fibres* were a frequent and abundant component; they were often fully differentiated and normal in appearance, despite their lack of innervation and their functional uselessness, fig. 7, Plate 23.

In no instance did the differentiated structures in the growing implant include any components unrepresented in the injected fragments. The implanted tissues bred true. Teeth were developed only in experiments in which jaw tissue had been used. Implants of tongue yielded skeletal muscle, non-epidermal stratified epithelial cysts and mucous cysts, and never cartilage, bone, teeth, or epidermis. There was no evidence of any metaplastic transformation of the growing tissues.

C—The Amount of Growth and Differentiation in the Implants—The amount of growth which had occurred in the implanted tissues can be estimated roughly. The largest masses obtained (*e.g.*, in rats 9, 10, 25, and 31, which were killed in 4, 7, 8, and 9 weeks respectively) had each a volume of about 0·25 to 0·5 cc, *i.e.*, about 100 times the volume of tissue implanted. Allowing for the volume of accumulated keratin and secretions, it is probable that the increase in volume of living tissues was about fiftyfold. This rate of growth is of the same order as that of the intact organism; a 1·5 cm embryo of 2 gm weight becomes a young rat of 100 gm weight in about 8 or 10 weeks. Evidently, then, in the most successful implants, the rate of growth of the tissues approximated to that obtaining in the growing rat.

The degree of differentiation which had been attained by the implanted tissues was in all cases far in advance of that present at the time of implantation. This was evident by comparison with sections of sample embryos taken from each batch used. In a 1·5 cm rat embryo, the epidermal glands, hairs, teeth, salivary glands, mucosal epithelia, and skeletal muscle-fibres are as yet very incompletely differentiated. Yet, in experiments in which tissues of this degree of immaturity were implanted, these tissues had achieved full structural differentiation in periods of 4 weeks or longer. Evidently, differentiation had been retarded but little or not at all by subdivision of the tissues and by the strange environ-· ment in which they were placed, but had proceeded at rates not much different from those obtaining in the intact organism.

D—Secondary Changes in the Host Tissues—Foci of degenerating squamous epithelial cysts, masses of keratin or nodules of cartilage were present in some of the implants, and these were associated with phagocytic phenomena on the part of the host-tissues—the collection of fat-laden macrophages or foam-cells, and the formation of foreign-body giant-cells. These changes, which were prominent in cervical and meningeal implants, were relatively infrequent in those in the brain.

Save for the probable participation of microglia in the phagocytic changes just described, the central nervous tissue bounding growing implants exhibited little or no reactive neuroglial proliferation. Cartilage, bone, teeth, and cysts were often to be seen in immediate contact with brain tissue, which, however, appeared in most instances to behave passively towards the intruders, figs. 1, 3, 4, 5, Plates 22, 23.

The implants often lay partly within the lateral ventricle, and in several cases in which part of the ventricle had become occluded the remainder of the cavity had suffered hydrocephalic distension, *e.g.*, in rats 12, 21, and 24. In several specimens naked hairs projected into the ventricles, apparently from implanted fragments of epidermis on their walls or on the choroid plexus. Mechanical crowding of the brain by the growing implants was frequently present, notably in rats 10 and 25.

Some of the intracerebral implants had become adherent to adjacent parts of the skull; and in rat 9, the wall of a large epidermal cyst had fused firmly with the dura over a wide area of the posterior fossa, so that on separation of the main part of the mass this area of the skull appeared to be clothed by skin. Rat 25 suffered asymmetrical distension of the skull by the bulky implant.

In spite of the large size attained by many of the implants and their wide encroachment on and crowding of the brain, symptoms of disturbed nervous functions were rarely seen and most of the rats appeared quite well until killed. Rat 15 exhibited severe ataxia, which, however, was due to the implant having been introduced into the brain-stem and cerebellum; and the convulsive death of this animal in 3 weeks was probably caused by infection of the implanted tissues. The only other rat to exhibit symptoms was *No.* 10, which had an opisthotonic attack under the anæsthetic, and which had a large intracerebral mass and extreme crowding of the brain.

E—The Possible Use and Scope of the Method—The experiments described in this paper are but few, and do little more than show that in rats the brain is a particularly favourable site for the successful transplantation of many embryo tissues. The method might profitably be

used further in the study of several problems. By transplanting minced fragments of individual embryo tissues or organs, the capacity of each of these for independent survival and growth could be studied; of particular interest might be the study of the ductless glands in this way. By transplanting whole the primordia of particular structures from early embryos, *e.g.*, the cartilaginous primordia of various bones, the information obtained by the method of culture *in vitro* might be amplified. By transplanting whole recently fertilized eggs, morulæ, or blastulæ, it might prove possible to obtain organized growth and differentiation of these, and so to attack in a new way some of the problems of tissue correlations in embryonic growth.

Finally, a word must be said as to the nature of the growing masses of tissue obtained in such experiments. Many authors have described them as " experimental teratomas " or " teratoids." In my opinion, the implication in these names is unjustified. The genesis of teratomas can have little in common with the artificial conditions of such experiments as these. The masses obtained from implanted embryo tissues are not teratomas; they are merely differentiating grafts, the study of which, however, under various conditions may prove of much interest in connection with the many obscure problems of growth and differentiation of tissues.

SUMMARY

The technique and results of some experiments on the implantation of embryo tissues in the brains of rats are described. The brain appears to be a highly favourable soil for such implants, in which cartilage, bone, hæmopoietic bone-marrow, epidermis, mucosal epithelia, teeth, salivary gland tissue, and skeletal muscle grow readily and differentiate well. In the most successful implants the amount of growth approximates to that of an intact animal, and the differentiation of the various tissues also proceeds at a nearly normal rate. Reactionary changes in the nervous tissue of the host are slight; and, in spite of extensive encroachment on the brain by the growing implants, the nervous functions and

health of the host-rats are rarely affected. The method may well prove of wider value in the study of some of the problems of growth and differentiation, and some further experiments are proposed.

DESCRIPTION OF PLATES

PLATE 22

FIG. 1—Rat 39. A bony mass containing hæmopoietic marrow. Note the absence of reaction in the contiguous brain tissue. × 41.

FIG. 2—Rat 21. A lingual epithelial cyst, showing ridging of the epithelium and of the layers of keratin within the cyst, presumably corresponding to the papillæ of the tongue. To one side of the cyst is a fringe of choroid plexus in the ventricle, and to the other side is a lattice of striated muscle-fibres with interspersed fat-cells. × 41.

FIG. 3—Rat 4. A well-formed incisor tooth. (Dense iron-hæmatoxylin staining.) × 16.

FIG. 4—Rat 33. A well-formed molar tooth in which the radial structure of the dentine is clearly seen. × 21.

PLATE 23

FIG. 5—Rat 28. A tooth with its base abutting against brain tissue, with which the dental pulp has achieved vascular connection. × 33.

FIG. 6—Rat 26. A tooth in which details of structure are well seen. Note pulp tissue, the layer of odontoblasts, uncalcified and calcified dentine with radial structure, and the layer of ameloblasts and enamel. To one side of the tooth are seen three mucous epithelial cavities and two islands of cartilage. × 50.

FIG. 7—Rat 4. Striated muscle-fibres (iron-hæmatoxylin stain). × 416.

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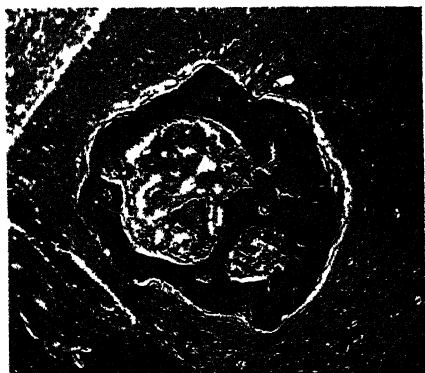
FIG. 1

FIG. 2

FIG. 3

FIG. 4
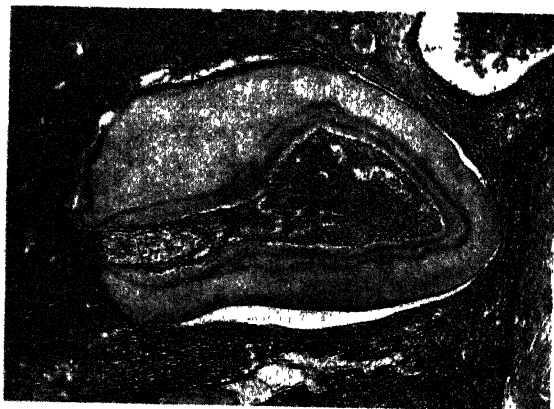
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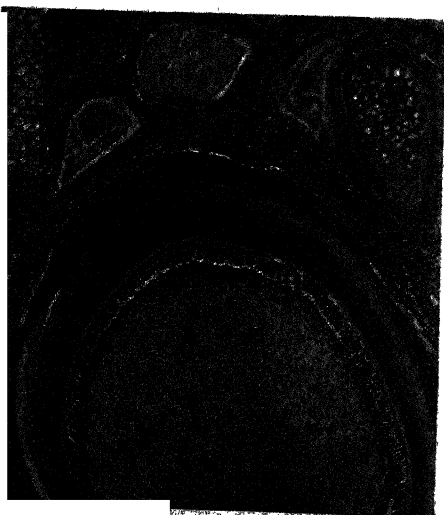
Fig. 6

Fig. 7

Experimental Studies in Insect Parasitism
III—Host Selection

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Introduction

Every parasite has a number, one or more, of host species which it will attack. The inclusion in this number of some species and the exclusion from it of others implies a process of selection on the part of the parasite which is usually known as " host selection."

That parasites do choose certain hosts from the larger number of animals available to them is a well-known fact; but why they choose those particular species and reject others remains unknown. That, indeed, is the principal problem of host selection—to discover the criteria used by a parasite in selecting its hosts.

The most recent discussion of the whole problem, that of Thompson and Parker (1927), leads to the sweeping conclusion that " the laws underlying the problem of host relations are not capable of expression in scientific terms or discoverable by scientific methods "; but it would be premature to accept so discouraging a view until more facts, and facts of a higher order of generality than are yet current in the subject, have been collected and examined.

A few of those facts are provided in the present paper. This study is an attempt to discover by scientific methods and to express in scientific terms some of the criteria by which ovipositing females of a small chalcid parasitoid, *Trichogramma evanescens*, choose their hosts.

The Meaning of Host Selection

Several distinct processes of selection may operate to make up or to restrict the host list of a parasite and, on this account, different writers have used the term " host selection " with quite different meanings. It is therefore necessary, first of all, to enumerate these selective processes and to make it clear which of them is here under discussion.

It is obvious in the first place, that in order to interact, the parasite and the host must meet, and that even parasites and hosts geographically and seasonally coincident may never meet unless they occur in the same œcological station. Now it is certain that some parasites, and probable that many more, are first attracted not to a particular host but to a certain type of environment. By selecting a particular environment in which to search for hosts, and neglecting other places, the parasite restricts the number of potential host-species that it will meet, and so limits the number of species on its list of hosts. This, if it is host selection at all, might be distinguished as an œcological selection of hosts.

Within the chosen environment the parasite may find many animals that might serve as hosts, but of these it selects only certain ones as suitable for attack. This process of selection is clearly a matter of behaviour and is, surely, host selection in its purest sense. It might be distinguished as the psychological selection of hosts.

Having found a host and attacked it, the parasite may yet be unable to parasitize it—the host may be too agile or its skin too tough. Even when a host has been attacked and parasitized, there is still a possibility that it may prove unsuitable, and the parasite be unable to develop upon its tissues. Examples are given below (p. 421). In such cases the host must be considered as selected by the parasite but removed from the list of suitable hosts by a process that might be distinguished as a physiological selection of hosts.

The confusion of these different processes—host finding, host selection, and host suitability—accounts for many of the contradictions that abound in the literature of host relations. Of the three, this paper is concerned almost exclusively with what has been called psychological selection. More particularly, since the data presented refer to *Trichogramma*, it has to do with the selection by adult parasitoids of hosts in which to deposit their young.

THE BEHAVIOUR OF *Trichogramma* WHEN SELECTING A HOST

Trichogramma evanescens is able to perceive its host, say an egg of *Sitotroga cerealella*, from a short distance away. It approaches, touches the egg with its antennæ, and immediately climbs upon it.

The parasite never oviposits without first subjecting its prospective host to a minute examination, and it is during this examination, presumably, that the selective faculty is exercised. If the host has already been parasitized, the inspection is brief, generally less than 5 seconds, and the host is then usually rejected (Salt, 1934, *b*). If, however, the

host has not been previously visited by another parasite, the examination normally continues for 15 to 30 seconds. Throughout this time the parasite moves forward and back and turns around and around on the host, tapping it with the antennæ. It is to be noted that *Trichogramma* never inspects the host by walking around it on the substratum, but invariably while standing upon it. As far as can be observed, the antennæ are the only organs used in the examination, and these, bent abruptly downwards from the scape, ceaselessly tap the surface of the egg.

When the host has been thoroughly inspected and if the parasite is satisfied, it raises its abdomen, disengages the ovipositor, and begins to drill through the chorion.

HOST SELECTION AND THE QUESTION OF BIOLOGICAL RACES

It is well known that within one species of animal there may be two or more biological races which, although morphologically identical, differ greatly in their behaviour. In the case of parasites this difference may take the form of a tendency to attack different kinds of hosts. Obviously, therefore, before the host relationships of a parasite can be critically studied, its biological races must be taken into account.

Marchal (1927, *a* and *b*) found two natural strains of *Trichogramma* occupying the same garden. They differed in colour, in their method of reproduction (arrhenotokous and thelytokous), and in the hosts they normally attacked. The thelytokous strain, which Marchal called *T. cacæciæ* because in nature it attacked only the eggs of *Cacæcia rosana*, would attack *Mamestra brassicæ* and *Ephestia* sp., normal hosts of the other race; but the arrhenotokous strain, *T. evanescens*, could not be induced to attack eggs of *Cacæcia rosana*. Marchal concluded that there are strains of *Trichogramma* having the status of races or of elementary species and differing, among other things, in the hosts normally selected for attack.

It is clear, then, since different races of *Trichogramma* probably exist, that in critical experiments on the host selection of this parasite care must be taken to use a pure strain.

The strain of *Trichogramma evanescens* used in the present work was originally obtained from Professor Hase of Berlin. It was reared as a stock culture for at least 60 generations on eggs of *Sitotroga cerealella*, possibly with some contamination by *Ephestia kuehniella*. When, at this juncture, it was decided to use the parasite for experimental purposes, a single female was removed from the stock culture. The progeny of this female, reared exclusively on eggs of *Sitotroga cerealella*, form the

principal experimental strain. It has now (December 12, 1934) been reared as a pure strain on *Sitotroga cerealella* for 98 generations. At the 20th generation a few adults were removed from this culture and placed with eggs of *Ephestia kuehniella*. This secondary strain has now been reared exclusively on *Ephestia kuehniella* for 78 generations.

Since eggs of *Ephestia kuehniella* are larger than those of *Sitotroga cerealella*, females of *Trichogramma evanescens* reared on *Ephestia* are larger and lay more eggs than females reared on *Sitotroga*. Apart from this difference in size, no morphological difference between the two strains has been observed.

However, although these two strains are derived from one original female, and although no morphological difference seems now to exist between them, it remains to be determined whether, having been confined for so many generations to different hosts, they have become in any degree fixed to those hosts; whether, in other words, they have become differentiated into biological races.

Experiments on this point were conducted as follows. Fifty eggs of *Sitotroga cerealella* 0 to 24 hours old were fastened in the alternate squares of a graph-paper disc (for technique, see Salt, 1934, *a*) and 50 eggs of *Ephestia kuehniella* also 0 to 24 hours old in the intervening squares. One hundred eggs were thus arranged 1/10 inch apart in 1 square inch, precisely as with a " standard " dish. It is to be noticed that the two host species were quite equally exposed to the parasite. One female parasite, 2 to 4 hours old, was put on the group of eggs and the dish placed in the incubator at 25° C for 8 hours. The parasite was then removed and the dish returned to the incubator to develop. Larvæ emerging from the unparasitized eggs were removed at frequent intervals because newly hatched *Ephestia* larvæ will eat other eggs, both parasitized and unparasitized. The resulting parasitism was noted on the fifth and subsequent days, and the emergence of both the parasites and the host larvæ recorded.

Such an experiment was first performed with five parasites from the stock culture previous to the foundation of the pure strain. They parasitized hosts as shown in Table I.

This experiment continued for 24 hours instead of only 8, so that the results cannot be strictly compared with those of the experiment that follows. They are given, however, as being the only record of the host preference of the original stock.

The only conclusion to be drawn is that these individuals of the original culture showed a distinct, though not an overwhelming, preference for eggs of *Ephestia*.

TABLE I—HOST PREFERENCE OF THE ORIGINAL CULTURE

Parasite	*Sitotroga* eggs parasitized	*Ephestia* eggs parasitized	Total eggs parasitized
1	11	18	29
2	11	20	31
3	13	19	32
4	22	22	44
5	27	31	58
Total	84	110	194
Ratio	43·3%	56·7%	

The next experiment was performed with the 63rd and 43rd generations, respectively, of the pure *Sitotroga* and *Ephestia* strains. Ten females of each strain were used with the results shown in Table II.

TABLE II—HOST PREFERENCE OF THE 63RD AND 43RD GENERATIONS, RESPECTIVELY, OF THE PURE *Sitotroga* AND *Ephestia* STRAINS

Parasite	*Sitotroga* strain			*Ephestia* strain		
	Sitotroga eggs parasitized	*Ephestia* eggs parasitized	Total	*Sitotroga* eggs parasitized	*Ephestia* eggs parasitized	Total
1	0	0	0	6	5	11
2	11	5	16	5	6	11
3	7	12	19	7	7	14
4	10	10	20	7	8	15
5	9	12	21	7	13	20
6	7	15	22	7	16	23
7	11	12	23	11	18	29
8	10	15	25	13	20	33
9	12	14	26	10	23	33
10	11	15	26	15	27	42
Total	88	110	198	88	143	231
Ratio	44·4%	55·6%		38·1%	61·9%	

From this experiment the following conclusions can be drawn: (1) The two strains, reared 63 and 43 generations on their respective hosts, had not become fixed to those hosts. (2) The two strains had not even developed a preference for their respective hosts, since both preferred the eggs of *Ephestia*. (3) The preference for eggs of *Ephestia* shown by the strain reared on *Sitotroga* was less than that shown by the strain reared on *Ephestia*.

The *Sitotroga* strain, which is principally used in the following experiments, had not in 63 generations developed a dependence upon *Sitotroga*

cerealella, or even a preference for that host over a strange one. The parasites used in the experiments described below were all from generations subsequent to this. It can therefore be taken as assured that, although biological races of *Trichogramma* may exist, the parasites used in the present study are of a pure strain as regards their selection of hosts.

OBJECTS ACCEPTED AS HOSTS BY *Trichogramma evanescens*

Hase (1925) lists 65 hosts of *Trichogramma evanescens*, by which he means hosts not only attacked but actually parasitized and which yielded a new generation of adult parasites. His list includes 53 Lepidoptera, 6 Diptera, 3 Coleoptera, and one each of the Hymenoptera, Hemiptera, and Neuroptera; and at that overlooks a record of Gatenby (1917, p. 153) of *T. evanescens* parasitizing eggs of dragonflies (Odonata). It is clear from this that *Trichogramma evanescens* is a polyphagous species; but even this list by no means exhausts the number of hosts.

When not only true hosts but also objects accepted and attacked by the ovipositing females are considered, the list can be greatly extended. Hase himself (p. 185) observed eggs of *Conorhinus megistus* (Hemiptera), *Trogoderma granarium* (Coleoptera), *Musca vomitoria* and *Stomoxys calcitrans* (Diptera), *Pulex irritans* (Siphonaptera), *Habrobracon juglandis* (Hymenoptera), and several spiders attacked and apparently parasitized, but in these eggs the *Trichogramma* was unable to develop (physiological selection).

In the course of the present study, the following objects have been accepted as hosts and attacked by ovipositing females of the *Sitotroga* strain of *Trichogramma evanescens*.

Eggs of Sitotroga cerealella *Olivier* (Lepid., Gelechiidæ)—This is the standard host on which the parasite is most easily reared. Usually one but occasionally two parasites emerge from a single host egg. I have observed *Sitotroga* eggs examined, accepted, and attacked in the following wide range of conditions.

Eggs not yet laid, dissected from the body of the moth, were readily attacked and in many cases produced fully formed adult parasites.

Eggs ranging in age from 0 to 120 hours old at 25° C appear to be equally suitable for rearing the parasite.

Eggs 120 to 132 hours old at 25° C, in which the *Sitotroga* larva was fully formed and to be seen moving inside the egg-shell, were accepted and attacked and in some cases produced adult parasites.

Empty egg-shells, from which either *Sitotroga* larvæ or *Trichogramma* adults had already emerged, were frequently observed to be examined and attacked.

Black eggs, five days after parasitization and containing full-grown larvæ or young pupæ of *Trichogramma*, were accepted. Judging from the short duration of the attack, it is probable that eggs were not laid (*cf.* Salt, 1934, *b*, p. 464); none could be found in the four which were microscopically examined. Care was taken to observe whether the emergents from these hosts were the result of the first or of the second attack because, if they had come from the second, the parasite would have acted as a hyperparasite of its own species. From each of 30 examples, however, the original parasite emerged normally five days after the second attack.

Black eggs containing adult *Trichogramma* ready to emerge are often examined and attacked by parasites already emerged in the stock cultures. It has not been determined whether an egg is actually laid. The attack has no effect on the adult occupant which, in all of the observed cases, emerged normally a few hours later.

Dead eggs, killed by exposure to heat or by long refrigeration or which for some other reason fail to develop, are readily accepted and parasitized. Eggs killed by heating all shrivelled before the parasites could develop, but eggs killed by prolonged refrigeration produced adult parasites.

Larvæ of Sitotroga—Larvæ of *Sitotroga* just emerged from the egg and either living or killed by momentary emersion in hot water were given no more attention than any obstacle in the path of the parasite if they were left in their usual position. If, however, dead larvæ were folded in the middle so as to form a mass of about the same size and shape as an egg, they were treated very differently. This especially occurred if two dead larvæ were intertwined so as to form a somewhat larger mass. Several of these were examined intently by the parasites for periods of 20 to 60 seconds in the manner that normally precedes oviposition. Five such masses, one of a single larva and four of two larvæ, were accepted and attacked. In one case the *Trichogramma* egg was found in the thoracic tissue of the dead larva. All the larvæ dried and shrivelled before any parasites could develop.

Lumps of Flour—Small globular masses of flour that have been retained in the sieve with the host eggs are frequently attacked in the stock cultures. This fact has already been noticed by Evans (1930, p. 109).

Glass Globules—Tiny glass globules of varying sizes but all slightly larger than *Sitotroga* eggs were eagerly accepted. For long periods the parasites vainly tried to drill into these strange hosts.

Seeds of Lobelia—To avoid the labour of making glass globules, seeds of Lobelia were tried. They were eagerly attacked.

Globules of Mercury—To permit variation in the size of the " host " and consequently a determination of the size limits, globules of mercury were exposed to the parasites. They attacked them readily.

Particles of Sand—Sand was passed through bolting-silk sieves so as to provide particles in a range of sizes. Suitable sizes were immediately accepted by *Trichogramma* females.

Cylinders of Glass—Cylindrical pieces of glass rod, of suitable dimensions, were attacked.

Crystals of Calcium Carbonate—The rhomboidal, almost cubical crystals of calcium carbonate of a suitable size were accepted as hosts and attacked.

Fragments of Glass—A piece of glass was shattered; pieces of a suitable size were obtained by means of sieves; and the most angular fragments were selected under the microscope. These angular fragments were readily attacked.

Eggs of Ephestia kuehniella *Zeller* (Lepid., Phycitidæ)—A common and suitable host. Usually one but sometimes two parasites emerge from a single egg.

' *Eggs of* Agrotis pronuba *Linn.* (Lepid., Caradrinidæ)—Eagerly attacked. Two parasites often develop in one egg.

Eggs of Lycæna icarus *Rott.* (Lepid., Lycænidæ)—Eagerly accepted but sometimes parasitized with difficulty on account of the hard and deeply sculptured chorion. Parasites emerged normally.

Eggs of Lepidoptera—Eggs of *Galleria mellonella* Linn. (Galleriidæ); *Pieris rapæ* Linn. (Pieridæ); *Epinephele tithonus* Linn., *E. hyperanthus* Linn., and *Pararge megæra* Linn. (Satyridæ) were attacked and produced adult parasites.

Eggs of Phytonomus posticus *Gyll.* (Coleop., Curculionidæ)—Six eggs already containing nearly fully developed larvæ were immediately

accepted by the parasites. Only one produced a fully developed adult; two yielded small under-developed parasites; and from three there were no emergents (partial physiological rejection).

Eggs of Bruchus obtectus *Say* (Coleop., Bruchidæ)—In the course of experiments described below, 93 eggs of *Bruchus obtectus* were attacked and in many, if not in all, *Trichogramma* eggs were actually laid. Adult parasites emerged from only 3; in most of the remainder the parasite died during its larval stage, in a few it succumbed when a young pupa (almost complete physiological rejection).

Eggs of Tenebroides mauritanicus *Linn.* (Coleop., Ostomidæ)—The few eggs available were attacked by *Trichogramma* females but produced neither *Tenebroides* larvæ nor parasites (complete physiological rejection).

Eggs of Muscina stabulans *Fall.* (Dipt., Muscidæ)—Nineteen eggs were observed to be accepted and attacked, and in some cases, at least, an egg was actually laid. From the 15 eggs left to develop neither *Muscina* maggots nor adult parasites emerged (complete physiological rejection).

No attempt has been made to increase this list to include many particular objects. It is intended merely to illustrate the different types of objects accepted as hosts by females of *Trichogramma evanescens*.

HOST PREFERENCE

The objects listed in the preceding section as having been attacked by ovipositing females of *Trichogramma* were accepted by the parasite in the absence of other hosts. The parasite was faced in each case with the necessity of attacking these objects or nothing. Such a selection is clearly of a very coarse nature. A much better knowledge of what the parasite requires in a host can be obtained from a study not of the objects it will accept, but of those it prefers to accept. The greater part of this paper, therefore, has to do with preference.

In the experiments summarized in Table II above, it was shown that *Trichogramma evanescens* prefers eggs of *Ephestia* to those of *Sitotroga*. The next, and important, step is to determine whether this preference can be analysed as the choice of certain definable properties of the *Ephestia* egg, or whether it is a preference for specific properties as yet indefinable.

Visually, eggs of *Sitotroga* and *Ephestia* differ most markedly in size, shape, and in the sculpture of the chorion (fig. 1, *a* and *b*). *Sitotroga* eggs are smaller than *Ephestia* eggs. The former are spindle-shaped,

about twice as long as broad in the middle; the latter are much stouter, broadly ellipsoidal, only about 1¼ times as long as broad. The chorion of *Sitotroga* eggs bears a distinct pattern of raised ridges and rather deep pits; while that of *Ephestia* eggs, although it bears a pattern, is comparatively smooth. To the touch these two species differ in the hardness of the chorion, the shell of *Sitotroga* eggs being harder and more brittle than that of *Ephestia* eggs. Since they almost certainly differ chemically, the eggs probably also differ in the odour that emanates from them.

In preferring *Ephestia* to *Sitotroga*, *Trichogramma* selected the larger, rounder, smoother, softer-shelled host. Can the host preference of the parasite be attributed to one of these physical properties, or must it be left vaguely attached to the chemical and indefinable specific qualities of the *Ephestia* eggs ? Observations of the behaviour of the parasite when confronted with other combinations of hosts will give a clue.

The eggs of *Bruchus obtectus* (fig. 1, *c*) are like those of *Sitotroga* in being spindle-shaped; but they are like those of *Ephestia* in being larger than *Sitotroga* eggs and in having a smooth chorion. Moreover, the *Bruchus* eggs are slightly, although only a little, larger than *Ephestia* eggs. Since *Trichogramma* does not develop normally in eggs of *Bruchus*, it is impossible to perform experiments similar to those described above using eggs of this species, but the difficulty can be avoided by observation experiments. A card of 50 *Bruchus* and 50 *Ephestia* eggs was prepared, eggs of the two species being arranged alternately in one square inch of the graph paper as usual. A single female parasite was then introduced and its movements watched and recorded as in experiments previously described (Salt, 1934, *b*, p. 462) until 25 attacks had been observed. Four such experiments were performed involving a total of 100 ovipositions. Two parasites attacked 12 *Ephestia* eggs and 13 *Bruchus*; one 11 *Ephestia* eggs and 14 *Bruchus*; and one 10 *Ephestia* eggs and 15 *Bruchus*. In all, 45 eggs of *Ephestia* and 55 of *Bruchus* were attacked. In each case and in the total, then, the parasites showed a slight preference for the eggs of *Bruchus*.

Eggs of *Agrotis pronuba* (fig. 1, *e*) are like those of *Sitotroga* in having a hard chorion which is thrown up into ridges, but they are nearly globular in shape and are much larger than the eggs of either *Sitotroga* or *Ephestia*. Observation experiments comparable in every way with the preceding were performed in dishes having 50 *Agrotis* eggs and 50 *Ephestia* eggs arranged alternately. Twenty-five ovipositions were observed by each of eight parasites. The eight females all preferred *Agrotis* eggs and of the 200 ovipositions observed, 146 were in eggs of *Agrotis* and only 54 in those

of *Ephestia*. The parasites showed a decided preference, then, for the eggs of *Agrotis*.

In the three sets of experiments so far described—*Sitotroga* versus *Ephestia*, *Bruchus* versus *Ephestia*, *Agrotis* versus *Ephestia*—the host preferred was first rounded then elongate, once rough and again smooth, at one time soft-shelled at another hard-shelled. It is clear that the parasite has no marked preference for any of these qualities. In each case a different host with different indefinable specific characters was chosen. It would not appear that *Trichogramma* is much concerned with them. In every case, however, the larger host was the one preferred. Is size, then, the important factor in the preference? This question can be answered by experiment.

The eggs of both *Sitotroga cerealella* and *Ephestia kuehniella* vary considerably in size, and it is possible under a binocular dissecting microscope to separate groups of large and small eggs. This fact affords an opportunity of testing the supposition that the preference of *Trichogramma* for *Ephestia* is a size preference. If it is a size preference, small *Ephestia* eggs pitted against large *Sitotroga* eggs should not be so much preferred as the normally larger *Ephestia* eggs pitted against the normally smaller *Sitotroga* eggs, while large *Ephestia* eggs pitted against small *Sitotroga* eggs should be even more preferred. If, on the other hand, it is a preference for other qualities, it should not be affected by the change in relative size.

Experiments on this point were conducted as follows. From a collection of *Sitotroga* eggs less than 24 hours old, the 50 largest and the 50 smallest examples were selected visually, under a microscope. From the remaining hosts, medium-sized examples were obtained by taking 60 at random and then rejecting the largest and the smallest individuals until the required 50 remained. A collection of *Ephestia* eggs was similarly treated. Large, medium, and small eggs of each species were thus obtained, and were arranged in three dishes as in previous experiments, the large *Sitotroga* alternating with the small *Ephestia*, the medium *Sitotroga* with the medium *Ephestia*, and the small *Sitotroga* with the large *Ephestia*. It should be noticed that only the very largest eggs of *Sitotroga* are as large as small *Ephestia* eggs, and that the average size of the large *Sitotroga* eggs used in these experiments was therefore slightly less than that of the small *Ephestia* eggs pitted against them.

Each dish was exposed to a single female parasite at 25° C for 8 hours. For these experiments females of the *Ephestia*-strain were used because their apparent preference for *Ephestia* eggs is greater (Table II) than that of the *Sitotroga*-strain, and might be thought more difficult to

alter. Ten such experiments were performed, with the results shown in Table III.

The middle group of medium-sized eggs of each species sets a controlled standard of 41·1% *Sitotroga* accepted to 58·9% *Ephestia*. The acceptance of *Sitotroga* in the other two groups was sufficiently higher, 47·9%, and lower, 32·8%, respectively, to show very definitely that the preference of the parasites was affected by the change in the relative size of the two hosts.

TABLE III—PREFERENCE OF THE *Ephestia*-STRAIN OF *Trichogramma* FOR *Sitotroga* AND *Ephestia* EGGS OF DIFFERENT RELATIVE SIZES

Large *Sitotroga* and small *Ephestia*			Medium *Sitotroga* and medium *Ephestia*			Small *Sitotroga* and large *Ephestia*		
Sitotroga	*Ephestia*	Total	*Sitotroga*	*Ephestia*	Total	*Sitotroga*	*Ephestia*	Total
11	13	24	10	14	24	9	14	23
15	11	26	12	12	24	8	20	28
13	14	27	9	16	25	6	23	29
12	18	30	12	15	27	15	17	32
17	14	31	13	18	31	9	24	33
20	16	36	14	20	34	9	24	33
17	21	38	19	17	36	10	26	36
19	19	38	14	23	37	14	23	37
16	25	41	12	28	40	14	27	41
20	23	43	16	25	41	16	27	43
160	174	334	131	188	319	110	225	335
47·9% *Sitotroga*			41·1% *Sitotroga*			32·8% *Sitotroga*		

It should be mentioned here that the variation in the preference of individual parasites apparent in Table III may actually represent fluctuations in the rigour with which the large and small eggs were selected by the experimenter.

The following two conclusions are to be drawn: (1) alteration of the relative size of the same two species of hosts affected the preference of *Trichogramma* for those hosts, the acceptance of each host-species varying directly with the size of that host relative to the other; (2) since the preference of *Trichogramma* for *Ephestia* over *Sitotroga* can be thus altered by a change in the relative size of the two hosts, it cannot be a specific preference for *Ephestia* and must be a preference for *Ephestia* simply as the larger host.

A final proof that size is the principal factor in the choice of hosts by *Trichogramma*, rather than any indefinable specific quality of an egg, is provided by two series of experiments using false hosts.

In the first series, 50 eggs of *Sitotroga* and 50 particles of sand larger than them (average volume 0·049 cu mm) were fastened in alternate squares of the graph paper. Females of *Trichogramma* were introduced and their attacks observed as usual until 25 had been made. Five parasites were used in each dish instead of only one because the parasites sometimes remain on one particle of sand for a very long time, vainly trying to drill into it. Four such experiments were performed so that a total of 100 attacks were observed. Of these, 66 were on particles of sand and 34 on the true hosts. Not only, then, were false hosts attacked in the presence of an ample supply of true and suitable hosts but also these false hosts, which the parasites could not even perforate to deposit their eggs, were more frequently attacked than the real hosts, that is, were actually preferred to them.

In the second series of experiments with false hosts, clean white sand was passed through fine sieves so that particles smaller (average volume 0·003 cu mm) than a *Sitotroga* egg and others larger (average volume 0·049 cu mm) were obtained. One hundred *Sitotroga* eggs were pasted on the graph paper in the alternate squares 2, 4, 11, 13, etc., of an area of 2 square inches. Four such dishes were prepared. One dish was left as a control. In the intervening squares, 1, 3, 12, 14, etc., of the second were pasted 100 of the small particles of sand; and in those of the third dish 100 of the large particles. Lobelia seeds were used to fill the intervening spaces of the fourth dish. A single female *Trichogramma* was introduced into each dish. After 8 hours in the incubator at 25° C the parasites were removed and the dishes returned to the incubator for development. The results of 10 such experiments are recorded in Table IV.

The parasitism in the control dishes and in those having small particles of sand was about equal. The parasites cannot have wasted much time, therefore, on the small sand grains. In the dishes having large particles of sand or lobelia seeds, however, the parasitism was reduced to less than half of the controls. The parasites in these dishes, therefore, must have paid more than half of their attention to, that is they must have preferred, the larger, false hosts.

From these two series of experiments the following conclusions can be drawn: (1) False hosts were attacked in the presence of an ample supply of true and suitable hosts. (2) The presence of small false hosts, smaller than the true hosts, caused no appreciable reduction of the parasitism; but the presence of false hosts larger than the true hosts produced a reduction of the parasitism to less than half of its normal value. (3) Lobelia seeds and particles of sand larger than the true hosts present

were actually preferred by *Trichogramma* to those true hosts; (4) since
a particle of sand or a lobelia seed cannot be supposed to have much
in common with an animal egg, the preference of *Trichogramma* for
them cannot be attributed to any indefinable quality of a host and must
be attributed to their larger size.

The question may now be asked, if *Trichogramma* attacks more of the
larger hosts, is it because the parasite actually selects the larger ones or
is it simply because the larger hosts are more readily found? This
question is answered by the following experiments.

TABLE IV—NUMBER OF *Sitotroga* EGGS PARASITIZED WHEN 100 WERE
ARRANGED ALTERNATELY WITH 100 FALSE HOSTS

	Hosts alone	Hosts and small sand	Hosts and large sand	Hosts and lobelia seeds
	0	0	0	0
	16	21	2	0
	21	21	2	0
	23	21	4	4
	23	22	6	5
	23	22	7	6
	24	23	9	9
	26	23	9	11
	26	25	17	20
	27	28	17	25
Total	209	206	73	80
Average	20·9	20·6	7·3	8·0

A standard selection dish of 50 small *Sitotroga* and 50 large *Ephestia*
eggs was prepared as usual. A parasite was put in and kept under con-
tinuous observation through a microscope, every egg touched, examined,
or parasitized being recorded in running notes. The experiment was
closed when the parasite had made 25 attacks. Four such experiments
were performed, with the results shown in Table V.

TABLE V—RESULTS OF 208 CONTACTS OF *Trichogramma* WITH SMALL
Sitotroga AND LARGE *Ephestia* EGGS

	Touched	Accepted	Rejected Paras. eggs	Rejected Unparas. eggs
Sitotroga	91	22	7	62
Ephestia	117	78	36	3
Total	208	100	108	

In the course of the experiment there were 208 contacts between the parasites and the hosts; 91 with the small *Sitotroga* and 117 with the large *Ephestia* eggs. This ratio of 117 to 91 may represent the greater ease of finding the larger hosts—the point will be dealt with by one of my students, Miss J. Laing, in a forthcoming paper on the finding of hosts by parasites. However, 7 of the contacts with *Sitotroga* and 36 of those with *Ephestia* were with eggs already parasitized and therefore not acceptable to the parasite. There were, then, only 84 contacts with unparasitized *Sitotroga* eggs and 81 contacts with unparasitized *Ephestia* eggs. Of the former, 22, or 26·2% led to oviposition; while of the latter, 78, or 96·3% led to oviposition. Only 3 unparasitized *Ephestia* eggs were rejected by the parasite, but 62 healthy *Sitotroga* eggs were refused.

It must be concluded, then, that the greater parasitism of large hosts is not, or is only in small part, due to the greater ease of finding them; but is due, for the most part, to an actual selection of the larger hosts.

To sum up: in all of these experiments—when eggs of *Ephestia* were exposed with those of *Sitotroga*, *Bruchus*, or *Agrotis*; when different relative sizes of *Sitotroga* and *Ephestia* eggs were exposed together; and when *Sitotroga* eggs were pitted against particles of sand or Lobelia seeds —it was the larger hosts in every case that was preferred by *Trichogramma*; in spite of the fact that, in some cases, the preference was for hosts (the *Bruchus* eggs) unsuitable for their progeny, in others for objects (the false hosts) in which they could not even deposit their eggs. No character other than their larger relative size was common to the hosts preferred as opposed to those rejected. Certainly no indefinable quality peculiar to a " host " was concerned in the selection. Finally, the selection observed was an actual preference, not merely a random proportion of the number of hosts found. From the whole series of experiments, therefore, it must be concluded that size is the principal criterion used by *Trichogramma* in its choice of hosts.

The Characteristics of Hosts Selected by *Trichogramma*

In the foregoing experiments, size was so overwhelmingly the most important criterion used by *Trichogramma* in its selection of hosts that all other factors that might have influenced the parasite in its choice were completely overridden and obscured. Other characteristics of the hosts have now to be discussed separately.

(1) *Position*—Many chalcid egg-parasites attack hosts which are imbedded in plant tissues and which protrude scarcely or not at all above

the surrounding surface. To test the reaction of *Trichogramma* to this condition, a pin was filed to such a shape that when it was pushed through paper it formed an elliptical hole 0·55 mm in length and 0·20 mm in greatest breadth, into which an egg of *Sitotroga* could be snugly fitted. One hundred such holes were made, $\frac{1}{10}$ inch apart, in one square inch of a graph-paper disc. An egg of *Sitotroga* was introduced into each of these holes and pushed down so that it was flush with the surface of the graph paper. A standard dish was made up from the same lot of eggs to serve as a control.

Twenty-five females of *Trichogramma* were introduced into each dish. They were removed from the control dish 1 hour later, and this dish was put into the incubator for the eggs to develop. The experimental dish with the sunken eggs was kept under continuous observation during the hour; it was then placed in the incubator for a further 6 hours; and at the end of this tqtal period of 7 hours the parasites were removed and the dish returned to the incubator for the eggs to develop.

In the course of the hour that the experimental dish was under observation, only one host was attacked, and during the further 6 hours, as shown by subsequent emergence, no others were parasitized. In the experimental dish, then, only one host was parasitized in 7 hours. In the control dish, however, as shown by subsequent development, 68 were parasitized in the exposure of 1 hour.

Observations made during the hour that the parasites were watched may be summarized as follows: in general there was no concentration upon or avoidance of the egg area, and parasites moved across it just as they did over the remainder of the surface. During the hour, seven examinations of hosts were made, three of one egg and one each of four others. Each of these five eggs was not pushed well into the hole but projected slightly above it, especially in the middle. The examinations were mostly brief (5 to 10 seconds) but one was of 20 seconds and the one followed by oviposition lasted 40 seconds. In these examinations the parasite seemed intent, as always, in determining the exact limits of the host. At the ends of the long axis there was usually a distinct gap between the host and the paper and the parasite had no difficulty in finding the end of the egg; but at the sides, where the egg fitted snugly against the paper, the tapping antennæ went off the host on to the paper as though not recognizing the difference. In at least two cases this led to the parasite rather losing than rejecting the egg, for it moved to the side, still tapping its antennæ, until it had passed right off the egg on the paper. The host that was actually parasitized fitted fairly snugly at the sides but left a distinct cavity between itself and the paper at each

end. The parasite stood entirely on the egg, orientated longitudinally, and parasitized it nearly in the middle. This case shows that the parasites were anatomically capable of laying in all these hosts; because this egg, though slightly, was not greatly raised, and in any case the parasite stood on the surface of the host only, not touching the paper on either side.

Since, as shown by this one case, the parasites were physically capable of attacking the sunken eggs, the great difference in parasitization in the two dishes can only be explained by supposing either (a) that the parasites fail to find hosts when they are sunk in the surface of the substratum; or (b) that the parasites reject such hosts as unsuitable. It appears that the former supposition is correct because one sunken host was accepted, and because other sunken hosts examined were lost rather than rejected. The experiment belongs, therefore, rather to the process of host finding than of host selection.

The following conclusions are to be drawn: (1) Twenty-five female *Trichogramma* placed on 100 sunken eggs parasitized only one in 7 hours; while an equal number of control females on 100 raised eggs parasitized 68 in 1 hour. (2) The sunken eggs, however, were not rejected as unsuitable; they were either not found by the parasite, or, if found, were in most cases lost again.

One essential characteristic of the hosts of *Trichogramma*, then, is that they shall protrude above the surrounding surface; but this characteristic has to do rather with the finding of the host by the parasite than with its acceptance or rejection.

(2) *Repellent Characteristics—Trichogramma* females running about in a dish avoid areas which have a gross smell, such as an area from which xylol or cedarwood oil is evaporating. They also avoid wet surfaces, on which they are apt to become stuck, or moving objects, which usually frighten them. It is not surprising, therefore, that the parasites will not climb upon wet or sticky or moving objects to examine them; or that they will not attack hosts that have been soaked in xylol or cedarwood oil. Such objects are not to be considered as rejected, because they are not examined; they are avoided as unsuitable places rather than rejected as hosts.

(3) *Odour*—There can be no similarity of odour among the different objects accepted as hosts by *Trichogramma*, and it can confidently be taken that the parasite is not led to accept hosts by their odour.

(4) *Texture*—Since *Trichogramma* invariably stands upon a host while examining and parasitizing it, the host must be sufficiently firm.

to bear the parasite. It may be very fragile (empty egg-shells of *Sitotroga*) or solid (sand grains) or actually a fluid (mercury globules) provided it is of such a nature that the parasite can walk on it.

The surface texture of the host seems to be of little account. The mercury globules attacked were quite smooth; the eggs of *Ephestia* finely reticulate; the eggs of *Sitotroga* and particularly of *Agrotis* and *Lycæna* coarsely sculptured. The hard surface of the glass and sand particles was attacked as readily and persistently as the thin shell of the true host eggs or the soft and yielding material of the lumps of flour.

(5) *Colour*—The accepted hosts include colourless objects such as the fragments of glass; silvery objects such as the mercury globules; white or cream objects such as the eggs of *Ephestia* or the fresh eggs of *Sitotroga*; pink or red objects such as the older eggs of *Sitotroga*; and dark objects such as the seeds of Lobelia, the eggs of *Agrotis*, and the black parasitized eggs of *Sitotroga*. The hosts selected by the parasite, then, seem to have no particular colour characteristic.

(6) *Absolute Size*—The smallest objects accepted by *Trichogramma* in this study were selected small *Sitotroga* eggs, 25 of which averaged 0·48 mm in length and 0·22 mm in greatest diameter. Reckoning these eggs as cylinders, their volume would be 0·018 cu mm. Since they are actually spindle-shaped, tapering to each end, their actual volume must have been less than this and of the order of 0·015 cu mm.

The largest objects attacked by the *Sitotroga*-strain, principally used in this study, were globules of mercury. The largest of these, having a diameter measured from above of 4·64 mm and a weight of 0·4195 gm, had a volume of 30·94 cu mm.

These two figures, obtained from a small strain of the parasite, do not necessarily set the full limits of size of hosts accepted by the species; but further discussion of the dimensions of the host is left for a later paper in which they will be discussed relative to the size of the parasite.

(7) *Shape* (fig. 1)—The list of accepted " hosts " includes spherical globules of mercury; ellipsoidal bodies such as the eggs of insects, lumps of flour, seeds, and glass beads; cylindrical rods of glass; and angular objects such as crystals of calcium carbonate, particles of sand, and fragments of shattered glass. It would appear from this list that, provided its size were appropriate, an object of any shape would be accepted as a host.

This, however, is not true if volume be taken as a measure of size. Cylindrical pieces of glass rod, 0·16 mm in diameter and 1·5 mm long,

fulfil the requirements of volume, having a capacity of over 0·03 cu mm, but they were not accepted as hosts. On the other hand, pieces 0·22 mm in diameter and 0·8 mm long, also having a volume just over 0·03 cu mm, and exposed to the parasites at the same time, were accepted. The different treatment of these two groups is easily explained. To be of any use the host must be able to contain the progeny of the parasite, which will be much the same size as its parent. Now, although their volumes were about equal, the former pieces of glass were too thin to have accommodated a *Trichogramma* within them, while the accepted cylinders could have contained one easily. In brief, to be acceptable to *Trichogramma*, a host must be of such a shape and size, that is of such dimensions, that it could contain the parasite.
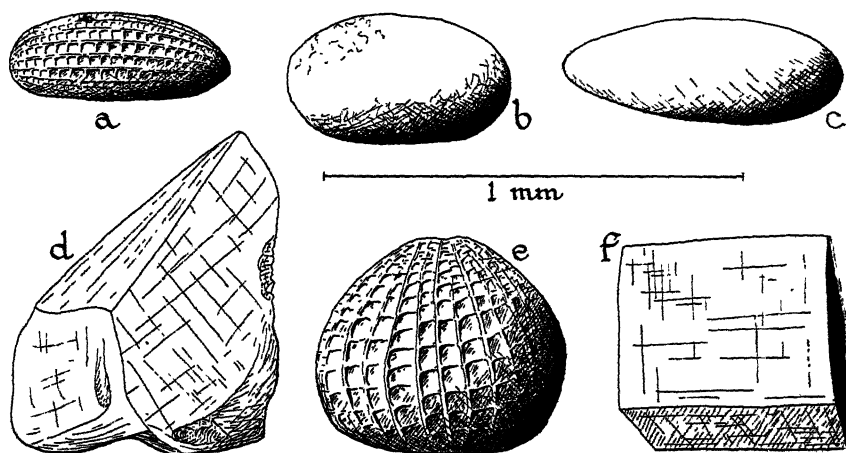
Fig. 1—Shape of objects accepted as hosts: *a*, egg of *Sitotroga*; *b*, egg of *Ephestia*; *c*, egg of *Bruchus*; *d*, fragment of glass; *e*, egg of *Agrotis*; *f*, crystal of calcium carbonate

Further, however, not any dimensions that will contain the parasite are acceptable in a host. Cylindrical pieces of glass 0·22 mm in diameter and 0·8 mm long were attacked by *Trichogramma*, as stated above; but pieces of the same diameter and 1·2 mm or more long were not accepted. It appears that just as there are maximum absolute dimensions, which fix the size, so there are maximum relative dimensions, which fix the shape. This whole question is to be discussed in a future paper on the relative dimensions of the parasite and the host. For the present it will suffice for a working definition to say that the shape of the host must be such that no one dimension is more than four times as great as another.

(8) *Definition of the Objects accepted as Hosts*—In summary, then, it appears necessary, if *Trichogramma* is to find its host, that it shall be

raised above the surrounding surface; if the parasite is to approach, that it shall not have a grossly repellent odour; and if the parasite is to examine .it, that it shall be unmoving, dense enough to be stood upon, and not be wet or sticky. These preliminary conditions satisfied and the examination begun, any object of a volume lying between 0·02 and 30 cu mm, of a shape that will contain the parasite, and having no dimension more than four times greater than another, may be accepted by ovipositing females of *Trichogramma* as a host.

DISCUSSION

The purpose of this study has been to discover some of the criteria by which ovipositing females of *Trichogramma* select their hosts. As a result of the work the most important of those criteria has been discovered (p. 427) and it has been found possible to give a working definition in physical terms of the objects accepted by *Trichogramma* females as hosts (p. 431).

Two points are to be emphasized: that *Trichogramma* does not select its hosts capriciously, but by means of certain criteria; and that those criteria are capable of statement in scientific terms. In these experiments the parasite did not arbitrarily attack one lot of objects and neglect another lot. Provided that certain requirements were satisfied, any object was accepted as a host and attacked. Moreover, in no case studied in the present research did the parasite select its host on qualities of an indefinable nature. The criteria used in selecting objects for attack were always physical and definable.

The principal interest of this result lies in the field of insect behaviour. From their discussion of the general problem of host relations, Thompson and Parker (1927, p. 33) conclude: " Pursuing the analysis of the process of food or host selection one finds, that this process occurs as if the ,suitability of substances as food or as hosts, obviously *not in itself per-ceptible*, were deduced by the animal from the perceptible qualities, though we have no reason to suppose that any conscious judgment occurs in the animal mind. This conception which corresponds exactly to the classical idea of instinct is the only one which seems to fit all the facts of the case." The behaviour of *Trichogramma* in the present study leads to a different view. Ovipositing females, not coerced by deprivation of suitable hosts but actually in the presence of true and acceptable hosts equally accessible to them, habitually attacked false and unsuitable hosts. Moreover, when the latter fulfilled the single condition of being larger than the true hosts available, the parasite not only attacked them,

but actually numerically preferred them. The classical theory of instinct, defined as the ability of the insect to recognize that which is suitable for its purpose, will not fit the facts of this case. Far from being a blind but purposeful instinct about which science has nothing further to say, the selection of hosts by *Trichogramma* is a phase of its behaviour capable of experimental analysis and of statement in scientific terms.

From the point of view of population studies on *Trichogramma*, three results of the present work are of interest. The experiments with false hosts show that the parasites waste their time on such objects, thus reducing their reproductive rate. The females in the dishes in which there were large sand-grains and lobelia seeds (p. 425) spent hours attempting to drill into these objects, to the partial neglect of the true hosts present. In the 8-hour period of the experiment the number of their progeny was thus reduced to little more than a third of its normal value, from 21 to only 8.

The acceptance by *Trichogramma* of unsuitable hosts in which, nevertheless, it actually deposits eggs, wastes not only its time but also its progeny. In the experiments in which eggs of *Bruchus* and of *Ephestia* were exposed together (p. 422), the parasites made 100 ovipositions. Of these, 45 were in *Ephestia* eggs and produced 41 adult parasites in the next generation. The other 55 ovipositions were in *Bruchus* eggs and yielded only three parasites in the next generation. The *Bruchus* eggs attacked by the parasite were killed, of course, like those of *Ephestia*, so that only the same number of hosts survived; but the population of *Trichogramma* in the next generation (44) was less than half of what it would have been (91) had all the eggs been laid in the suitable hosts.

The habit of *Trichogramma* of preferring the larger hosts present has still another effect on the subsequent ratio of the parasite and host populations. In very large hosts, *Trichogramma* frequently lays more than one egg. Therefore, when very large hosts such as eggs of *Pieris* are exposed with smaller hosts such as eggs of *Ephestia*, the parasite progeny are not distributed broadly, one in each, but less widely, two or three in each large host. This is not superparasitism, and the number of parasites emerging in the next generation is the same as if the eggs had been distributed separately; but the number of hosts that escape to reproduce in the next generation is larger.

These three results have direct application to biological control operations. Both sand grains and small seeds are objects which *Trichogramma* probably meets in its normal environment; either might well be found adhering to the surface of a leaf after a wind. The parasite probably also finds unsuitable hosts. These laboratory experiments suggest that

the potential reproductive rate of *Trichogramma* in nature is considerably reduced by the attention it pays to false and unsuitable hosts. As for the general preference of *Trichogramma* for large hosts, the occurrence of large hosts of no great economic importance when the economic host is not available may be very useful to carry on the population of the parasite, but its presence at the same time as the economic host may, owing to the parasite's preference for it, seriously hinder the control measures.

Such considerations as these may explain the differences of opinion that have arisen among economic entomologists with regard to the value of *Trichogramma* as a control for certain insect pests. In the controversy at present raging in the West Indies, each side may be right in its own territory. If, in Antigua, more sand particles are blown on the cane leaves than in Barbados, or an unsuitable or a larger host than *Diatræa* is there available, *Trichogramma* may waste its time and progeny on the one island sufficiently to make it a less effective means of control than it is claimed to be on the other.

A minor point in connection with the material used in this work remains to be mentioned. In much of the work that has been done on host selection, insects have been presented with a strange food or with a choice of foods, and hunger was the urge that impelled them to make their choice. In such experiments the alternatives before the animal are acceptance of the offered food and starvation, at least temporary. Those conditions are severe. It may be of use to biologists, therefore, to point out that insect parasitoids afford experimental material for studies of host selection in which there is no fatal result to the selecting animal of a refusal or a wrong choice. *Trichogramma* females live for days without any apparent inconvenience in the entire absence of hosts, and some other parasitoids actually live longer when deprived of hosts than when placed with them.

SUMMARY

Ovipositing females of *Trichogramma evanescens* were observed to examine, select, and attack, besides a number of true hosts from which their progeny successfully emerged, several unsuitable hosts in which

their progeny were unable to develop, and a variety of false hosts, such as particles of sand and globules of mercury, in which they were unable even to lay their eggs.

Two strains of *Trichogramma* reared exclusively on *Sitotroga cerealella* and *Ephestia kuehniella* respectively for 63 and 43 generations, developed no dependence on or even preference for their respective hosts.

Both of these strains preferred eggs of *Ephestia*; but an analysis of the preference showed that it was not a specific preference for *Ephestia* but a preference for *Ephestia* simply as the larger host.

When two different kinds of hosts were simultaneously exposed to *Trichogramma*, in every case the parasite showed a preference for the larger of the two, even though the preferred host was in some cases unsuitable for the development of its progeny, in other cases a false host in which the parasite could not even lay its eggs.

From this experimental analysis and from a review of the characteristics of objects accepted by *Trichogramma* as hosts, it appears that the principal criterion used by ovipositing females of *Trichogramma* in the selection of their hosts is that of size.

Trichogramma evanescens does not select its hosts on qualities of an indefinable nature, peculiar to certain species of animals; but uses criteria that can be defined in scientific terms.

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The Relationship of the Blood Sugar Level to the Systemic Blood Pressure

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The marked hyperglycæmia which follows section of the brain stem through the pons in fasted rabbits (Donhoffer and Macleod, 1932) and in cats (Peterson, 1933) could not be observed in fasted rats although it occurred in recently fed ones (Bell, Horne, and Magee, 1933). In certain of the experiments with fasted rats, instead of a rise in the blood sugar level, a decided fall occurred, but the main feature of these results was that transection of the brain at any level did not markedly affect the blood sugar. This contrasts with the marked rise which invariably occurs in the rabbit, cat, or fed rat when the pons is involved. On further investigation of this peculiar behaviour of the fasted rat, it was soon found that although the blood sugar level did not rise markedly. as in the cat and rabbit, it sometimes rose slightly and still more frequently fell somewhat following decerebration. As the work progressed, it became apparent that the lesions which caused the blood sugar level to become lowered were also those which might be associated with a marked fall in arterial blood pressure, and accordingly the effects of low blood pressure *per se* on the blood sugar level were investigated. These effects are usually considered to be such as will raise rather than lower the sugar level, but it must be remembered that the observations from which this impression has been formed have as a rule been made on animals whose livers contained glycogen from which sugar can be formed by glycogenolysis. When the liver is free of preformed glycogen, however, and can produce sugar only by gluconeogenesis, the effects of low blood pressure are entirely different, as the present investigations show.

Methods

Albino rats and rats of the Lister strain were used after 24 hours' fasting, when the glycogen content of the liver is, on the average, 0·180% (Barbour, Chaikoff, Macleod, and Orr, 1927; Cori and Cori, 1928).

Decerebration through the pons or at a higher level was performed, under ether, by means of a thin wire blunted at the point and inserted through a trephined hole, or by the Schmidt method, as modified by Bell for the rat. The lower end of the medulla or the cord was approached through the occipito-atlantoid membrane or in a few cases through the cerebellum. After the experiment the head was preserved in formalin solution and the exact position of the decerebration ascertained later and marked on a photograph. Bleeding from the vessels of the dura was difficult to control, but otherwise there was usually little loss of blood. By using a small glass cannula with a flattened bulb filled with 3·8% sodium citrate solution, there was no difficulty in measuring the blood pressure, but kymograph records were not taken because the manometer had to be of narrow bore to minimize loss of blood into the tubing. The actual values, owing to capillarity, were 16% higher than those observed. Except where otherwise stated, the values given in this paper are those actually read on the manometer, the same instrument being used in all the rat experiments. Blood samples for analysis were collected from the femoral artery by allowing it to bleed into Scarpa's triangle previously sponged with oxalate solution, the blood being then quickly sucked into a narrow pipette—0·1 cc for estimation of sugar by the Hagedorn Jensen method and 0·3 cc for that of lactic acid by the Friedmann Cotonio and Shaffer method. The experimental error for the latter estimations was ascertained to be ± 0·006%. In experiments where artificial respiration was employed, the blood was carefully examined at each sampling to see that it was maintained in an arterial state.

RESULTS

As already stated, the blood sugar level was found sometimes to rise and sometimes to fall following decerebration, and in an endeavour to find out whether the exact position of the brain lesion determined the nature of this result, a large number of experiments were performed. The pons and cerebellum are very poorly developed in the rat and, in correlating the blood sugar changes with the level of decerebration, it was found convenient to distinguish only two regions in the brain, region I being the portion anterior to a line running from the anterior edge of the pons to the posterior edge of the posterior colliculus, and region II, the relatively long portion of the brain stem between this line and the spinal cord. As indicated in fig. 1, this bulbar region was further subdivided into smaller sections, numbered from before backwards. At one stage of the investigation, the behaviour of the blood sugar level seemed in some degree

subject to the position of decerebration, but later the fact became more and more apparent that some other factor was responsible. The curves of figs. 2, 3, and 4 are typical of the results obtained at this stage of the

FIG. 1

Decerebration just in front of the pons at zero
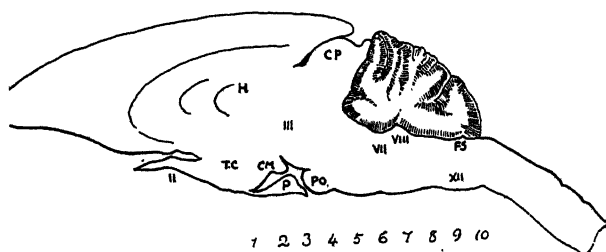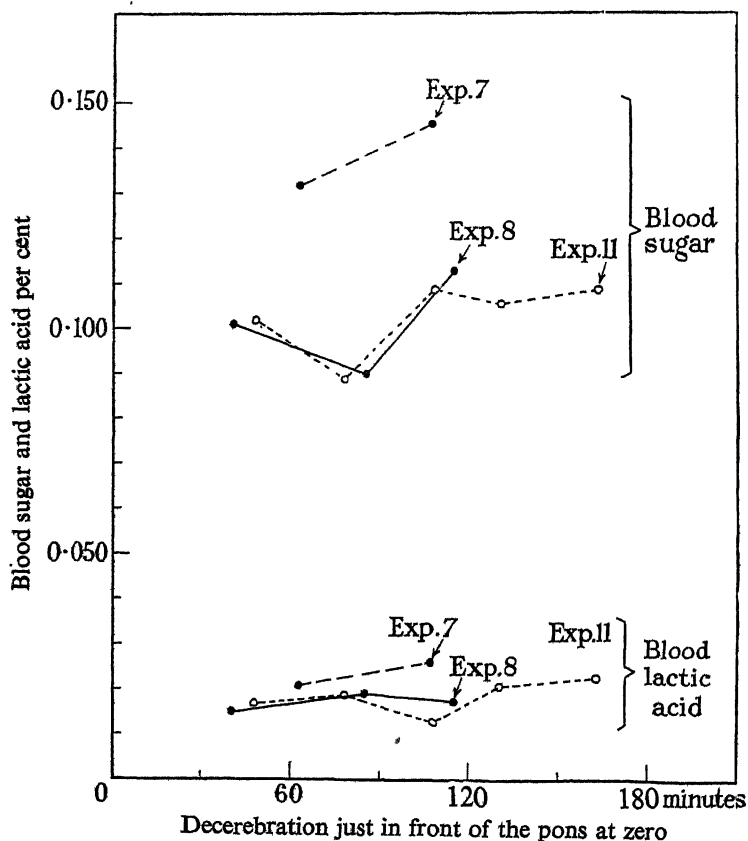
FIG. 2

investigation. When decerebration was performed in region I the results shown in fig. 2 were sometimes obtained; there was no significant change in the levels of sugar and lactic acid of the blood for periods

extending up to 3 hours after the operation. If this result were regularly obtained a valuable preparation would be available for studying, in the absence of anæsthesia, the effect on the blood sugar level of various experimental procedures. But this is not so, since, even when decerebration was definitely restricted to this region, the blood sugar level might either rise or fall. An example of the former effect is shown in fig. 3
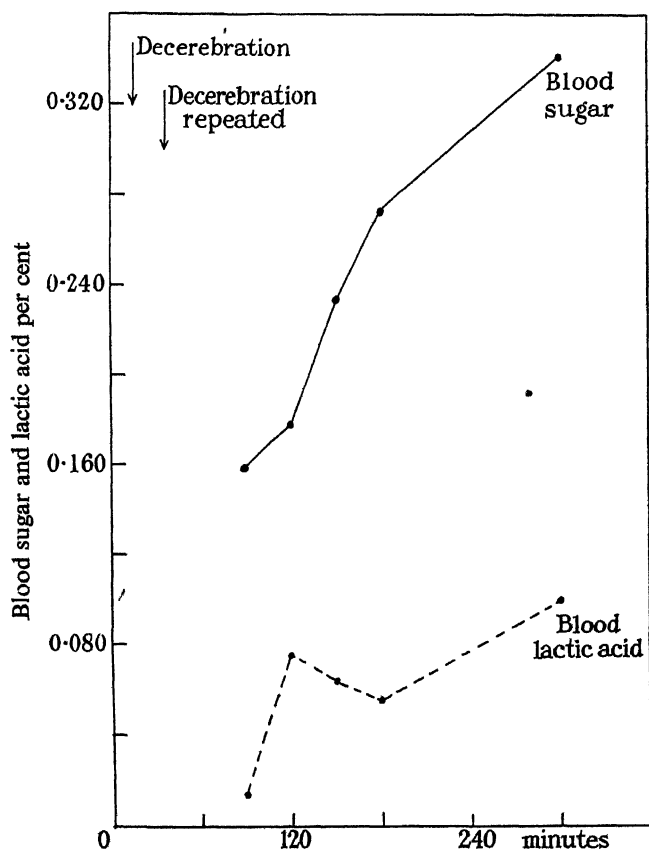
FIG. 3—Exp. 5a

and it will be observed that the lactic acid rose as well as the sugar. This animal was hyperexcitable after the decerebration and post-mortem examination showed that the brain had been cut further forward than usual. Bulatao and Cannon (1925) have shown that when the cerebral cortex is removed, leaving the subthalamic region intact, a hyperglycæmia occurs which they attribute to the removal of cortical inhibitory influences acting on the sympathico-adrenal chief nucleus situated in the subthalamic region. It may be that a similar decorticated preparation was

obtained in this instance. In no other experiment, involving a cut in region I, was so striking a rise in blood sugar obtained, but occasionally a less marked and temporary rise occurred, attributable probably to subthalamic overactivity.

In six experiments, results like those shown in fig. 4 were obtained. In all of these, for the purpose of obtaining an animal with normal blood sugar level which could be used for studying the effect of further lesions

FIG. 4

Decerebration just in front of the pons at zero
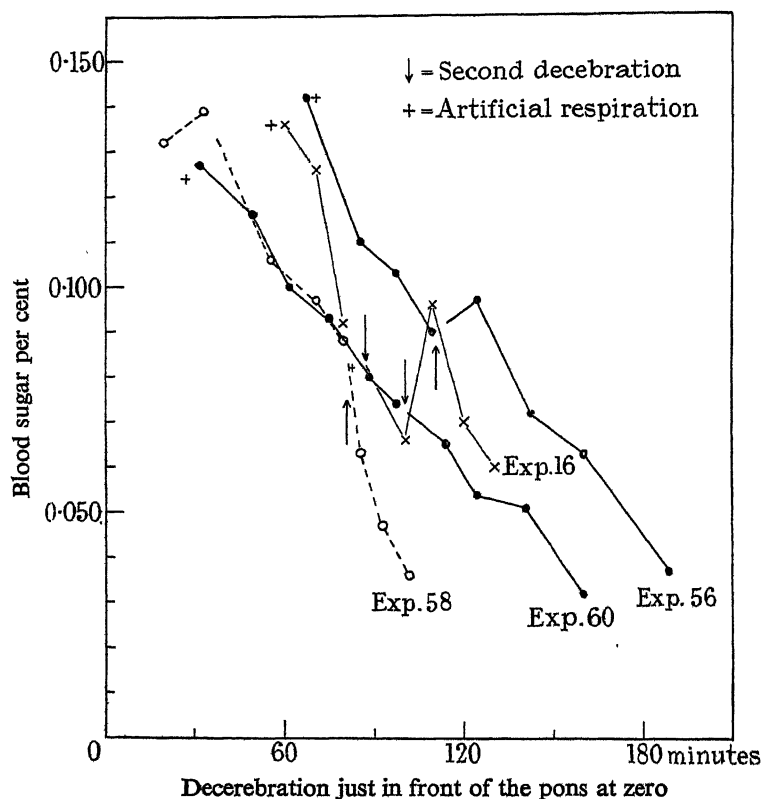
at lower levels of the brain stem, decerebration was first performed in region I (at zero time). In all cases the blood sugar fell after decerebration (the only exception being experiment 58 where there was a temporary rise), and a later decerebration in region II did not alter the rate of fall. One invariable characteristic of all these experiments was failure of breathing so that the animal had to be kept alive by artificial respiration. There were also 10 experiments in which decerebration was performed in region II, either exclusively or following decerebration further forward, and the curves of fig. 5 show how the blood sugar behaved in

three typical experiments in which the level was between the levels 3 and 4 of fig. 1.

It is unnecessary to give further evidence to show that in the rat with minimal liver glycogen at least some factor other than the level of de-cerebration *per se* has a prepotent effect on the blood sugar. An impression that this might be the blood pressure was formed from the observation that low blood sugar levels were more frequent in animals in which there had been considerable bleeding or shock during the preliminary operative procedures; for example, hypoglycæmia invariably developed after
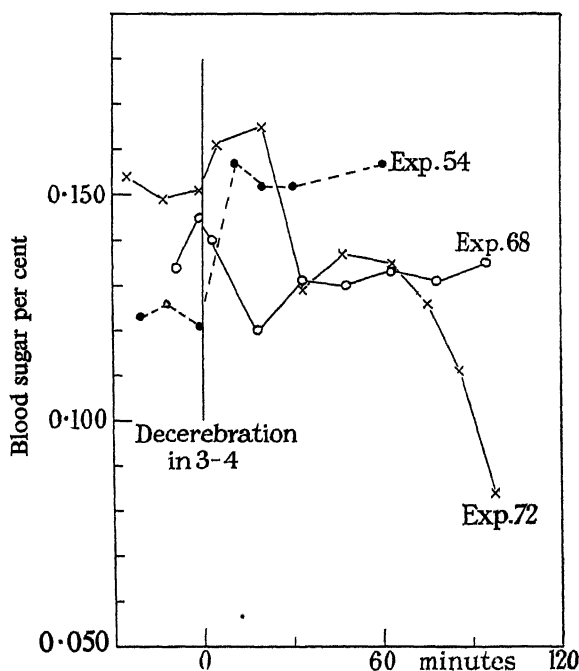
FIG. 5

decapitation, whereas it occurred less frequently after simple section of the cervical spinal cord.

The effects of decapitation are shown in fig. 6. In one of the curves it will be observed that the blood sugar remained stationary for some time (3 hours) after decapitation, but out of a total of 16 experiments this is the only one in which such a result was obtained. The fall of blood sugar per hour was between 0·028% and 0·058%, averaging 0·042%. It may be of interest to note here that the rate of fall of blood sugar in Mann and Magath's experiments (1924) on liverless dogs was 0·012 to 0·013%. The liver glycogen content of the rats at the end of the experi-

ments ranged between 0·024 and 0·048%, showing an average of 0·033%. In contrast with this, fig. 7 shows the behaviour of the blood sugar level after section of the spinal cord without decapitation.* It remained practically constant.
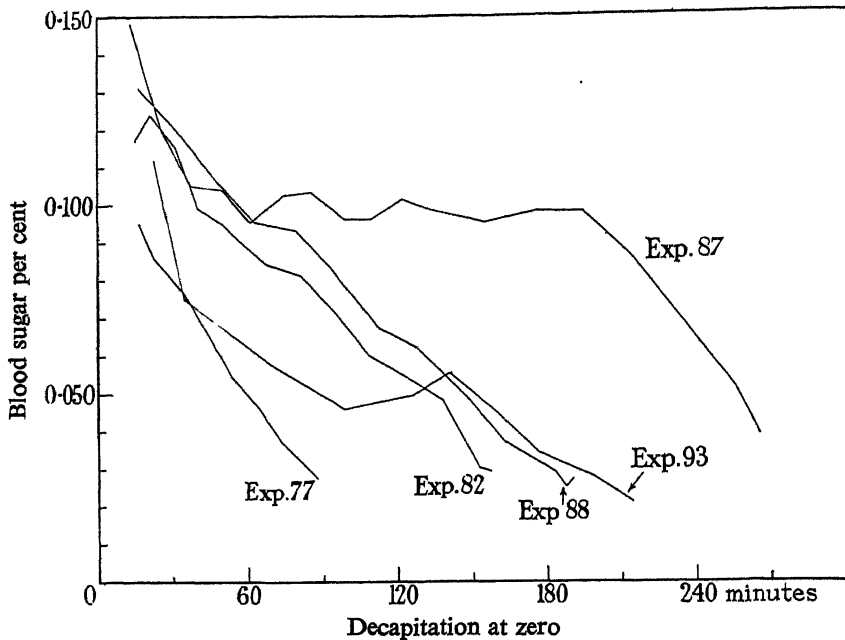
FIG. 6
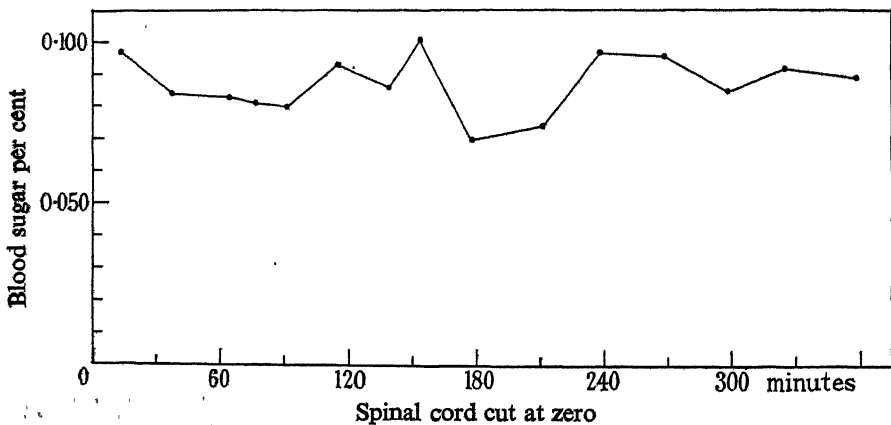
FIG. 7

* When the spinal cord was cut without decapitation, the animal was invariably decerebrated previously in region I.

These experiments reinforced the impression that the factor primarily determining the fall in the blood sugar level was not so much that nerve pathways from a glucogenic centre had been severed, but rather that the arterial blood pressure had fallen very low. The pressure was, therefore, measured at regular intervals and the results of four typical experiments are shown in fig. 8. After decapitation the blood pressure was very low (40 mm Hg or less), and in each instance the blood sugar level fell more or less steadily during the 3–4 hours of survival of the preparation. In two experiments (91 and 93) the blood pressure partially recovered and
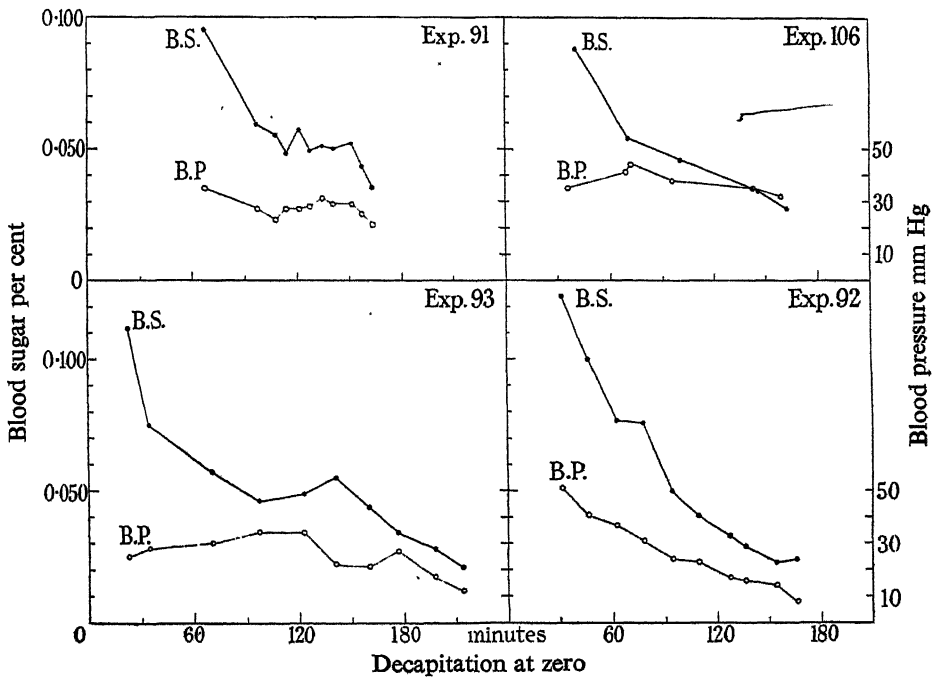
FIG. 8

it is possibly of significance that there was a concurrent decrease in the rate of fall of the blood sugar level. In other words, a certain degree of parallelism exists between the two curves.

It may be advantageous here to consider the results of other experiments in which the blood pressure was measured. In the first of these, decerebration was performed in region I and was accompanied by a stoppage of natural breathing. The blood pressure, which was 65 mm Hg 25 minutes after the operation, rapidly fell to 40 mm and then more slowly to below 20 mm Hg, this fall being accompanied by a decrease in blood sugar percentage which continued for the 3½ hours during which the animal

survived (see fig. 9). In several experiments in which decerebration in region I did not cause the blood pressure to fall to a low value, the blood sugar level was also maintained, as is shown in the first portions of the curves of experiments in fig. 10 (experiment 94) and fig. 11.

This evidence strongly supports the view that the fall in blood sugar level in the decerebration experiments results from a lowered arterial blood pressure. Further examination shows that the hypoglycæmic effect does not set in until the blood pressure has fallen to a fairly definite
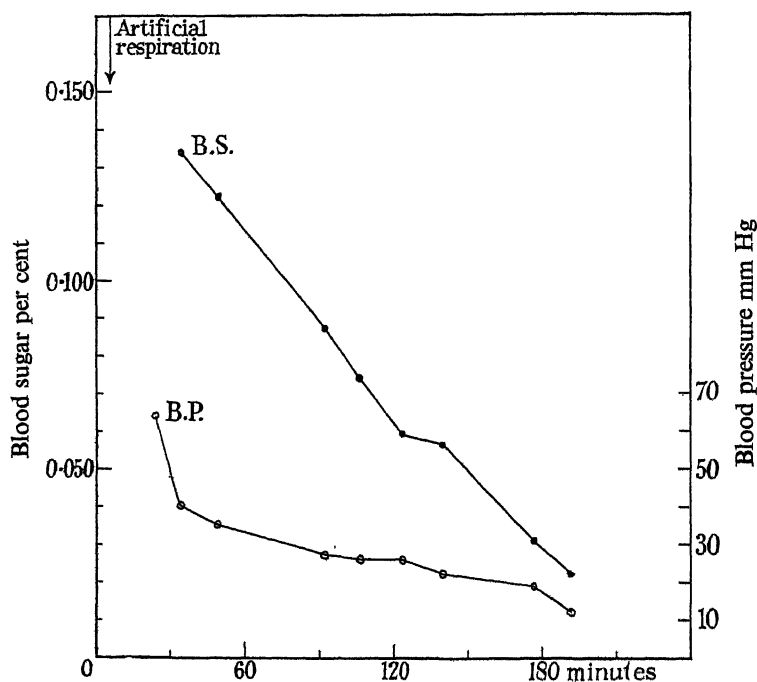
FIG. 9— Exp. 97: Decerebration just in front of the pons at zero

low level which, in the rat at least, is very close to 40 mm Hg (corrected value 46 mm). In other words, there would appear to be a critical level of blood pressure below which the blood sugar begins to fall. Further evidence for this is shown in the curves of fig. 10 relating to an animal of which the spinal cord was cut without decapitation. These results probably explain previous ones such as those shown in fig. 7, in which the spinal cord had been cut. It is probable that in this experiment the blood pressure did not fall so far as to reach the hypoglycæmic critical level.

These observations naturally led to an examination of the effect on the blood sugar level of lowering the blood pressure by other methods; the first to be tried was injection of acetylcholine. In the experiment shown

in fig. 11 a large dose of this drug was injected intramuscularly, causing the blood pressure to fall rapidly. In the next experiment (fig. 12) acetylcholine was given intravenously by a continuous injection pump, at first at the rate of 0·6 mg in 11 minutes, but later at a much slower rate, viz., 1·1 mg per hour. The injection was continued for 2 hours 40 minutes and the blood pressure fell to the 40 mm level almost at the outset where for some time it remained without further fall, becoming
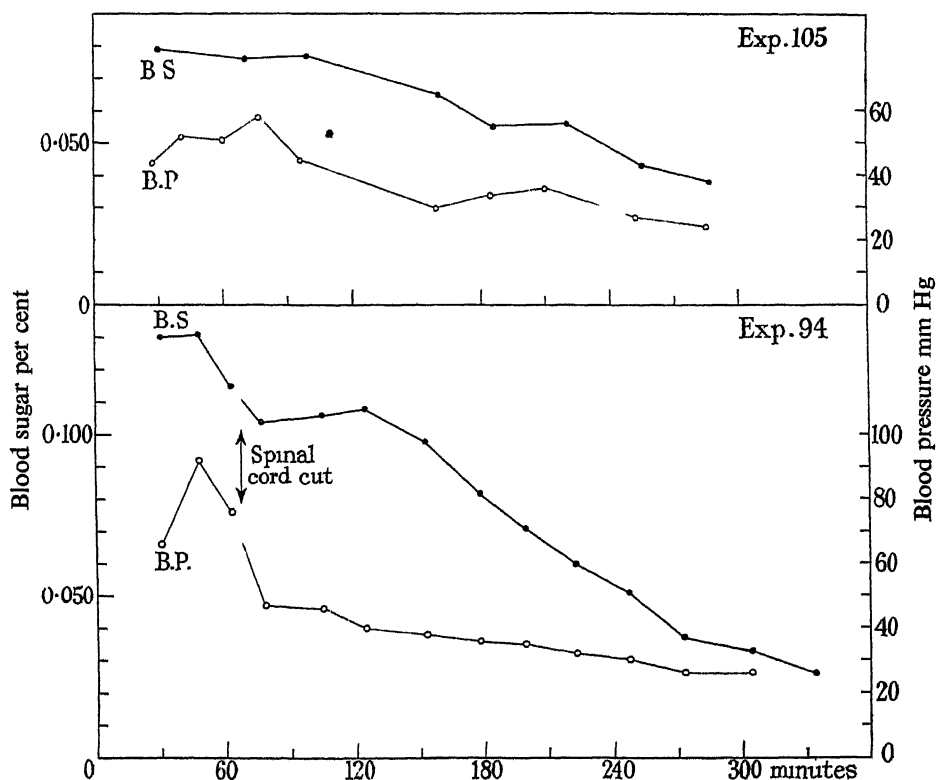
FIG. 10—Exp. 105: Spinal cord cut at zero.
Exp. 94: Decerebration just in front of the pons at zero

indeed temporarily raised above this level, probably because of the slowing down of the rate of injection. Later, however, it definitely fell below 40 mm and at this point the blood sugar concentration also began to fall, reaching 0·037% when the blood pressure was about 10 mm Hg. It is also worthy of note that the hypoglycæmia became pronounced long before artificial respiration had to be applied. It has to be added that this animal was under urethane, which probably accounts for the rise in the blood sugar level preceding the injection of acetylcholine.

The next step was to see how the blood sugar level would behave when, after being lowered by low blood pressure, the latter was raised above the critical level. The curves in fig. 13 show the results of two experiments in which the blood pressure was brought below the critical level by decapitation and was then restored by continuous intravenous injection of defibrinated rat blood. Although large volumes of blood
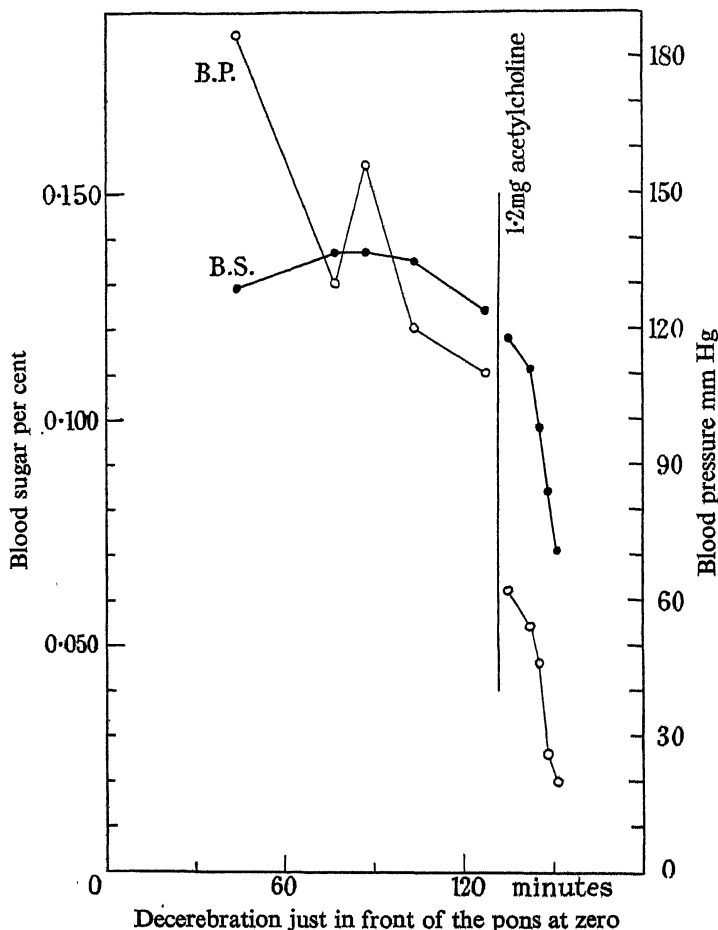
FIG. 11

(recorded in fig. 13) were required to restore the blood pressure, the sugar contained in the added blood was in itself not nearly adequate to account for the rise in the blood sugar level. Thus in experiment 103, 10 cc of blood were injected during the first period which lasted 1 hour 13 minutes, the rate of addition of glucose being about 10 mg per hour. It is obvious that such a small amount of glucose added to the circulation of a 300 gm

rat could not perceptibly affect the blood sugar level even under such abnormal circulatory conditions. It is of importance that the blood sugar level began to rise immediately the transfusion was started and before the critical level referred to above had been reached. It was observed that the pulse in the arteries immediately became stronger when the injection was started, so that the improvement in the circulation —in the mass movement of the blood—must have been more marked than the blood pressure would indicate. After the blood sugar level had been at 0·100% for 100 minutes—the blood injections being mean-
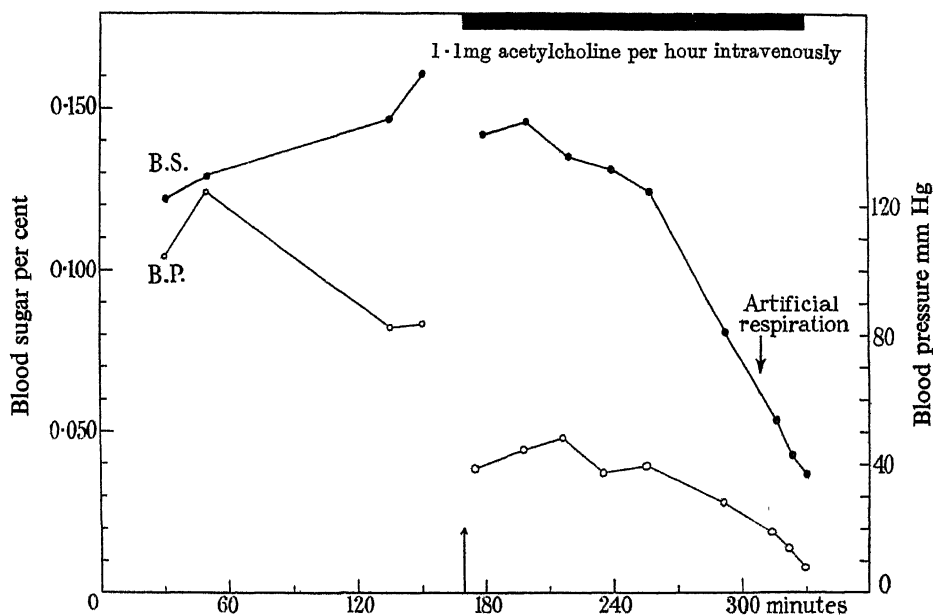
FIG. 12—Exp. 98: Urethane at zero

while continued with two short interruptions—the blood pressure began to fall in spite of the injection. Post-mortem examination showed considerable pulmonary oedema and the other viscera were markedly congested.

The results of experiment 108 are in the main similar to those of experiment 103, but two points deserve special notice: (1) the blood sugar level was maintained for at least 40 minutes after the transfusion had been discontinued, and (2) it then began to fall roughly parallel with the blood pressure. The amount of sugar injected with the blood in this experiment was 12·4 mg in 85 minutes during the first injections and 2·2 mg in 20 minutes in the second one. These again are amounts far too

small to account for the rise observed in the blood sugar level. That blood was circulating freely through the muscles and nerve centres in these preparations was evidenced by the fact that various nerve reflexes could be elicited almost to the time of death.

These are the only two out of a total of six experiments in which the animals lived sufficiently long to give dependable data. In the others, sudden death occurred, either because the blood pressure had become too
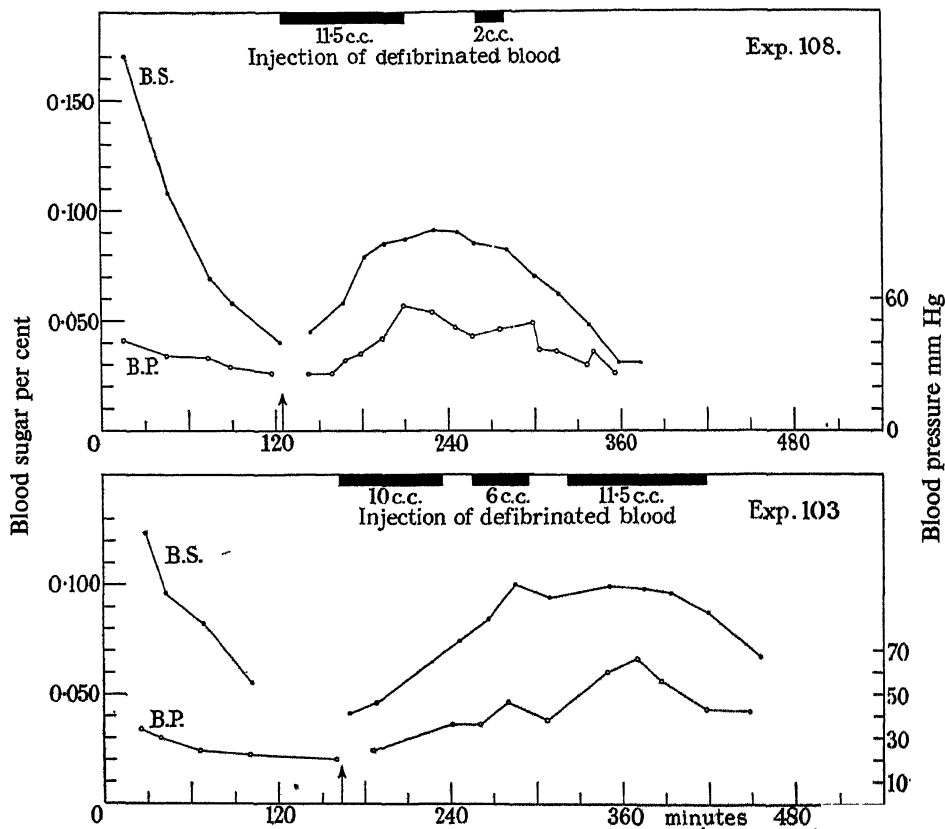
FIG. 13—Decapitation at zero

low before transfusion was started or because of some toxic effect of the added blood. On this account the transfusion was next performed using Tyrode solution with 8% gum arabic dissolved in it. The reducing power of this solution was 0·020%. The results of such an experiment are shown in fig. 14. It will be seen that the blood pressure rose very soon after the injection was started, but was not sustained when the injection was temporarily stopped when the animal exhibited convulsive movements.

Although it remained above the 40 mm Hg level, however, the blood sugar level soon began to fall. Since the blood had been diluted, it may be assumed that the actual rise in blood sugar percentage was greater than that indicated by the observed sugar concentrations.

The foregoing results are interpreted as indicating that the process of sugar secretion by the liver (gluconeogenesis) ceases when the arterial blood pressure falls below a certain critical level. This would appear to be about 46 mm Hg (corrected value). When the liver contains no preformed glycogen, as in the fasted rat, the cessation of gluconeogenesis results in hypoglycæmia.
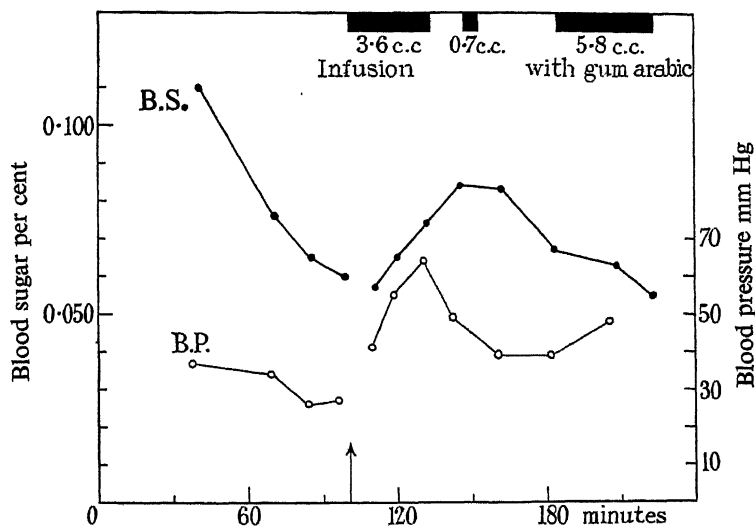
Fig. 14—Exp. 109: Decapitation at zero

It is interesting to compare fasted rats having little glycogen in the liver, with fed ones having high percentages. It is well known that in animals such as cats and rabbits in which the liver glycogen percentage is appreciable even after fasting, a marked fall in arterial blood pressure is associated with hyperglycæmia. But this had not so far been shown for the rat. Rats were, therefore, fed for 24 hours with cane sugar only, and it was found that the liver glycogen rose to about 7%. When the spinal cord in these animals was cut, the blood pressure immediately after the operation was usually below 40 mm, but for some time after the operation while the ether effect was passing off the pressure in some instances rose as high as 80 mm. This period of slowly rising blood pressure lasted, on an average, 20 minutes after which it fell steadily until the death of the animal which occurred in from 100 to 210 minutes
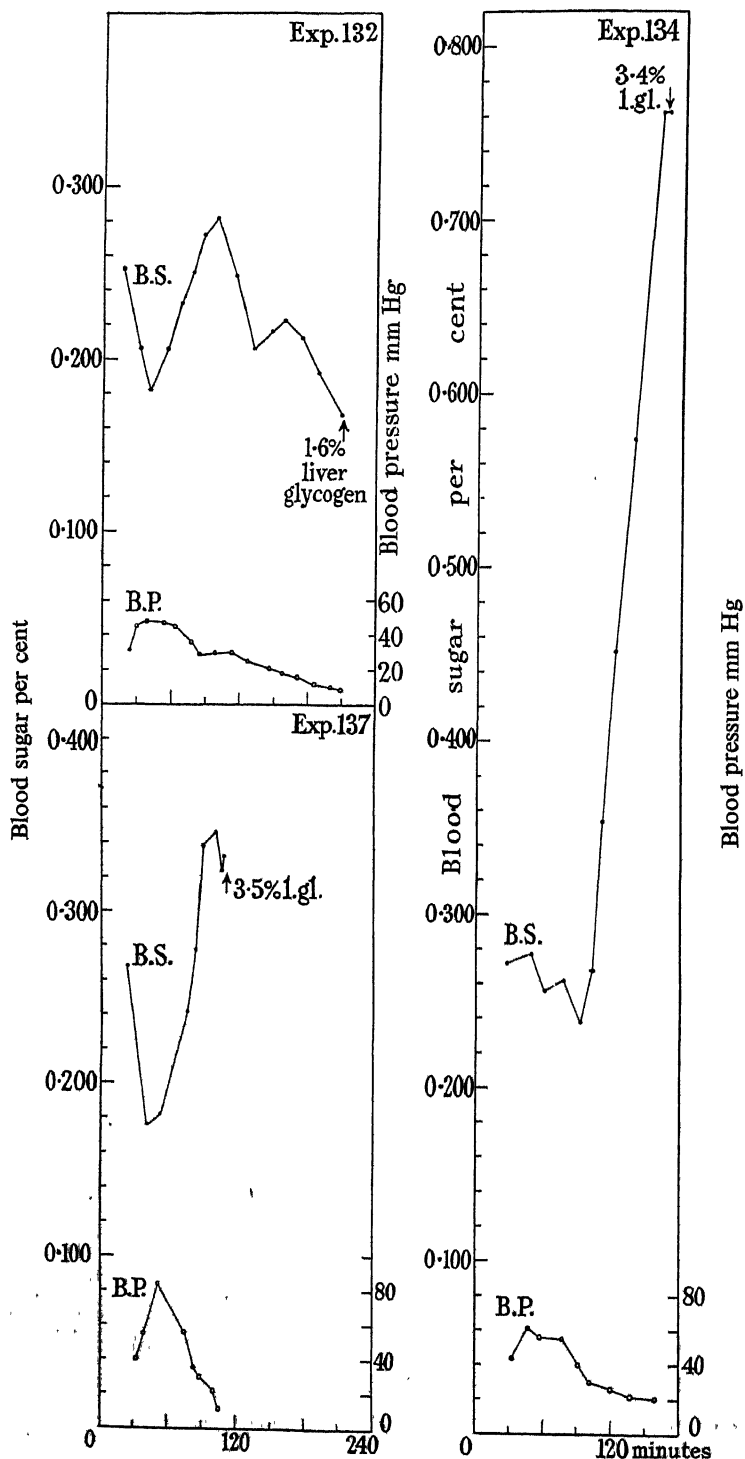
after the operation. The blood sugar curve fell more or less until the fall in the blood pressure set in, when it sooner or later rose very steeply as in experiment 134, fig. 15, or more gradually as in experiments 132 and 137. The results were therefore entirely different from those obtained on the fasted animal. They show that in the fed rat a fall in blood pressure to well below the shock level is associated with marked glycogenolysis, and in the fasted rat (and probably in the fed one, as well) gluconeogenesis becomes suppressed.

In other laboratory animals, such as the rabbit, it is very difficult, if not impossible, to rid the liver of glycogen by fasting, the only certain method being by administration of phlorrhizin. Experiments of this type have been performed in the four experiments, of which the results are shown in fig. 16. Phlorrhizin suspended in oil was injected subcutaneously for roughly 40 hours into rabbits which had been deprived of food the previous day. The animals had free access to water, but several of them did not take it and these were invariably in much poorer condition after the injection than those which drank freely. The blood sugar levels were very low in the dehydrated animals and high in the others. The liver glycogen was also (as can be seen in the chart) much higher in the animals which took water freely. Experiment No. 115 shows the effect of urethane alone on a rabbit in which the blood sugar was very low (0·065%). The blood pressure gradually fell to below the 40 mm level after which the blood sugar fell to 0·047%. In experiment 120, the animal was in good condition, the liver glycogen being 0·5% and the initial blood sugar 0·135%. Ether anæsthesia, followed at once by decapitation, brought the blood pressure very low. The blood sugar level at first rose somewhat, probably owing to glycogenolysis, but soon fell rapidly reaching 0·067%. In experiments 118 and 119 decerebration was performed under amytal. The results are much the same in both instances, the blood pressure rapidly falling to below the 40-mm level accompanied ultimately by falling blood sugar which in one instance reached 0·060% and in the other 0·040%.

These results confirm those obtained with the fasted rat in that a fall in blood pressure, however caused, to what is commonly known as the shock level (40 mm Hg) is accompanied by a marked lowering of the blood sugar level. It must be pointed out, however, that whereas in the rat the critical level of blood pressure is very close to 40 mm (corrected value 46 mm) it may vary somewhat in the rabbit. In this animal also the experiments show that when the blood pressure has fallen to a certain degree the blood sugar begins to fall also.

DISCUSSION

A close relationship between the blood sugar level and the blood pressure is seen in the rat, but it is also clearly evident in the rabbit. It has not as yet been clearly demonstrated for other animals, the difficulty
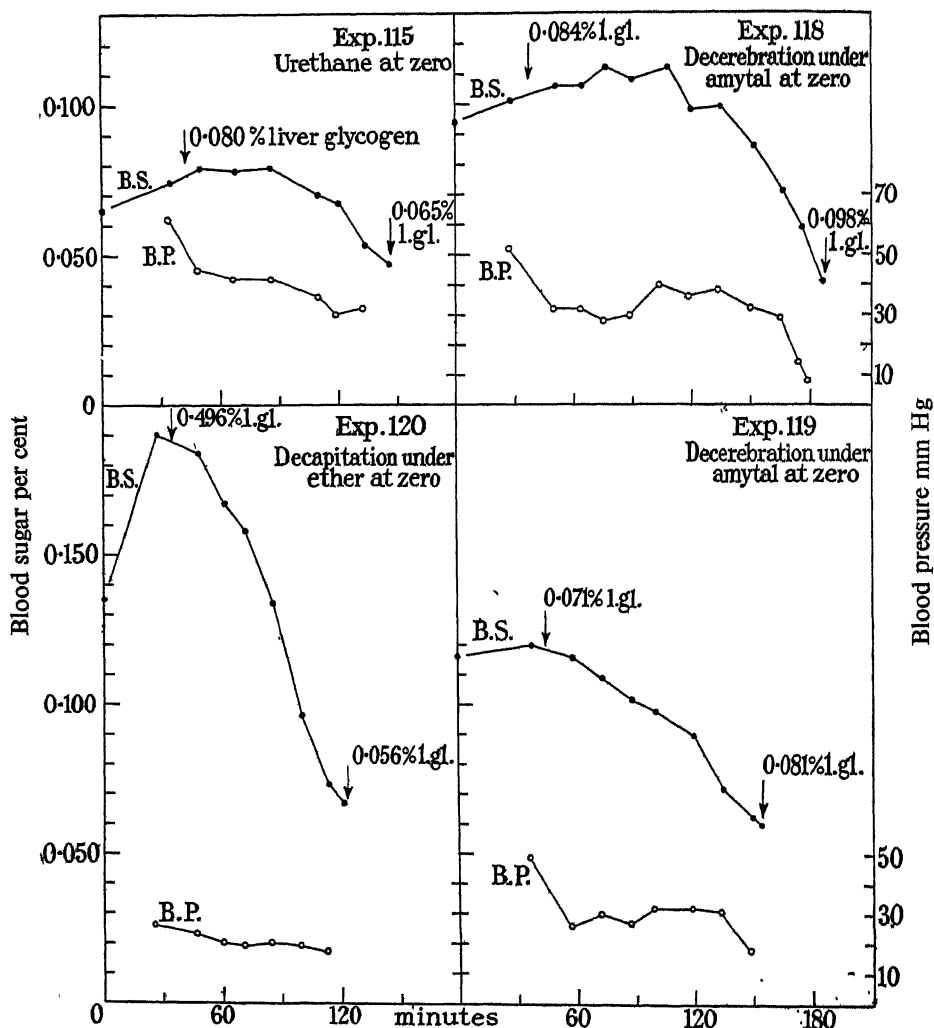
FIG. 16—In experiments 118–120 the kidney vessels were tied

being to find some method by which the liver can be made glycogen-free. For further investigation of the relationship between blood pressure and blood sugar it will be necessary to find some method for using larger animals, such as the cat. That the relationship between the two is

fundamental is shown by the experiments in which the blood pressure after having been temporarily lowered, was raised again by transfusion either of blood or gum saline. The recovery of the blood sugar under these conditions is very striking, but there is one noteworthy difference in the behaviour of blood sugar with rising blood pressure (due to transfusion) as compared with a falling pressure, namely, there is no evidence during transfusion that the blood sugar rise is related to any particular (critical) level of blood pressure. Indeed, the blood sugar curve began to rise immediately the transfusion started and it may be significant that the heart action was at the same time decidedly improved.

The relationship here shown between low blood pressure and hypoglycæmia has not hitherto been recorded by other workers, no doubt for the reason that they have used animals in which the liver contained glycogen. Donhoffer and Macleod in the light of their experiments on the effects of decerebration at various levels in fasted rabbits made the suggestion that there were, physiologically speaking, two varieties of glycogen: one, that deposited from absorbed carbohydrate, and the other, that resulting from gluconeogenesis. It is of interest that this hypothesis has recently been shown by Willstätter (1934) to be borne out by chemical investigation. The results of the present experiments also support this hypothesis in so far as they show that when traces of gluconeogenic glycogen alone are present, a fall in blood pressure has an entirely different effect from that which it has when abundant (storage) glycogen is present. The latter has been the case in our experiments with the rat fed with cane sugar as well as those of other workers who, in studying the effects of decerebrations. and decapitations, have, at least in some instances, measured the blood sugar when the blood pressure was falling to a low level. Hypoglycæmia has, however, never been observed in these experiments. The general impression among workers in carbohydrate metabolism is, indeed, that low blood pressure is usually associated with a rising blood sugar and we believe that the effect which the pressure will have depends entirely upon whether or not there is preformed (storage) glycogen in the liver. When there is, glycogenolysis occurs and the blood sugar level rises, when there is not, glyconeogenesis stops and the blood sugar falls.

The impression gained from the experiments on rats, but not so strikingly from those on rabbits, that this cessation of gluconeogenesis occurs at a certain critical level of blood pressure, is of interest. Such a critical level is known to determine glomerular filtration in urine formation, but in the present experiments we have evidence that a similar critical level of blood pressure is necessary for the performance of what would

appear to be a purely chemical process, namely, that of gluconeogenesis. The activity of nerve cells is also known to be readily affected by low blood pressure, but the activity of salivary and analogous glands appear to be less closely related to blood pressure. It is impossible in the light of present knowledge to explain the mechanism by which low arterial blood pressures brings about this effect. The most probable factor involved is the volume of the blood flow through the portal vein, and it is important in this connection to note that in recent experiments McMichael (1932) has demonstrated that the decrease of portal blood flow in a condition of low arterial blood pressure is as marked as that after clamping either the mesenteric arteries or veins in animals with a normal blood pressure.

The author gratefully acknowledges the constant guidance of Professor J. J. R. Macleod throughout this investigation.

SUMMARY

In the fasted rat, with liver glycogen reduced to a minimum, a fall in blood pressure below a critical value is accompanied by a rapid decrease in the blood sugar level. The same is true of the rabbit depleted of liver glycogen by phlorrhizin. For the rat the critical level is approximately 45 mm Hg.

On restoring the blood pressure of the fasted rat by transfusion of defibrinated blood or gum saline, the blood sugar level is re-established.

In the fed rat and non-phlorrhizined rabbit, in both of which there is ample liver glycogen, a fall in blood pressure below this critical level is accompanied by hyperglycæmia.

The fall in blood sugar in the animal with minimal liver glycogen is believed to be due to the suppression of gluconeogenesis consequent on the fall of blood pressure below the critical level.

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A Further Account of the Feeding Mechanism of
Chirocephalus diaphanus

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[Plate 24]

Introduction

During the last ten years Professor Storch and I have published a
series of papers on the feeding mechanism of the Branchiopoda. While
we agree on the whole and support the views of the earlier workers,
Lundblad and Naumann, there are certain fundamental points on which
we differ. Last year I published a lengthy descriptive and comparative
paper on the subject which summarized the position of the problem as
I saw it at the date of its publication, June 20, 1933. Since then two papers
have appeared, one by Lowndes (1933) on the feeding mechanism of
Chirocephalus, and the other by Eriksson (1934) on the feeding mechanism
of the Branchiopoda, in neither of which, unfortunately, is any reference
made to my last paper. This would have been of little consequence if
these scientists had accepted the views of other workers, but as they both
put forward entirely novel ideas the publication of their papers can
only lead to confusion in a problem which is already extremely difficult
to follow.   •

Lowndes's paper is relatively short and makes little reference to previous
work—in fact, Storch's work is ignored except for the inclusion of two
of his papers in the literature list. The treatment of the problem is that
of statement of new ideas with little, if any, argument as to why my views
are incorrect and often, and on the most critical points, without any
experimental or morphological evidence. ˙

Eriksson's paper, on the contrary, is very long with an immense litera-
ture list and a mass of historical detail. In the present paper I shall only
deal with that part concerning the Anostraca.

Eriksson gives a reasoned argument for every point, but it is clear
cannot have much faith in my views or in those of Storch. Unfortunately

there are several passages which show that, in referring to my paper on *Chirocephalus* (1928), he has either incorrectly translated or has failed to understand my views. As these cases include some of the fundamentals of the feeding process, this seriously detracts from the value of his criticisms and makes a reply wellnigh impossible. For example, in my description of the feeding currents of *Chirocephalus* I state that water is sucked from the median space between the two rows of limbs into the spaces between successive limbs, which I call the inter-limb spaces. These spaces are enlarged as the limbs move forward and as a result water is sucked into them. Now Eriksson, p. 87, states that, according to my description of the feeding mechanism, these inter-limb spaces are of little significance and the mechanism would work whether they existed or not. I cannot understand how he has deduced this from my papers, for I coined the words " inter-limb spaces " as a convenient term to denote the essential current-producing structure.

Then again (p. 88) he states that I have maintained that a structure, which by its movement in water produces a current, can affect particles floating near it independently of the water currents produced, whereas actually, as he correctly points out, such particles follow the resulting streaming of the water. Again, I am afraid I am unable to find any statement of mine that might be interpreted in this manner. If one assumes that floating particles can be moved independently of the currents in which they float it would be impossible to do anything more than make mere conjectures as to any feeding mechanism.

Despite this apparent disagreement, as far as I can see there is little real difference between the views of Eriksson and myself—at least as far as the Anostraca are concerned—with the exception of two points which I deal with in the present paper. On all other points he has accepted my views.

The main points on which it is certain Eriksson disagrees with me are in connection with the method of production of the oral food current and the functions of the labral glands. He supports my contention, and hence disagrees with Storch, that particles are carried to the mouth on a definite current, but maintains that it is produced as a continuous stream and not intermittent as I hold. The labral glands, he considers, produce a secretion which entangles surplus food so that it can be thrown away, not so that it can be eaten, which is the view I have put forward. However, I welcome his views on this point as he is the first worker who has seriously considered my suggestion that the labral glands produce a food-entangling secretion. Storch has always been non-committal on this point.

THE PRODUCTION OF THE ORAL FOOD CURRENT

I recently stated (1933, p. 274), " All workers agree that the long setæ on the edges of the basal endites of the trunk limbs constitute the retaining wall that keeps the food particles in the median space,"* and further, that the food so retained is transferred to the mid-ventral food groove *en route* for the mouth.

Now I maintained (1928, p. 811) that the food is carried to the mouth on a water current, while Storch maintains that it is swept forwards from limb to limb by the proximal setæ of the basal endites. I answered Storch at length (1933, p. 323) and pointed out that I demonstrated the existence of this orally directed current by experiment. My experiment consisted of injecting a solution of methyl blue so as completely to fill one of the inter-limb spaces of a captive *Chirocephalus*. Then it could be seen that, while the majority of the dye was thrown out posteriorly and laterally, a portion was shot forward *in a series of jerks* along the mid-ventral line, each jerk coinciding with the end of the backstroke of the limb, behind which the dye had been injected. Now while Storch, Eriksson, and Lowndes all dispute my results, only Lowndes has attempted to repeat my experiment, and he has confirmed it (1933, p. 1109). I therefore take it as established that there is an orally directed food current and that this is intermittent and not a continuous stream.

Eriksson disagrees with Storch and agrees with me so far as to admit the existence of the current, but he maintains (p. 62) that it is continuous and not intermittent. He states in a footnote (p. 63) " That Cannon describes the current in the mid-ventral groove as intermittent depends most certainly on the fact that, as he states (*wie er angibt*), the limb movements of the animal on which the observations were made, were very slow and irregular." Actually I stated (1928, p. 811), " If a *Chirocephalus* is fixed by its abdomen,† ventral side uppermost, to a piece of plasticine in a small dish of water *it will continue to function normally*." I admit that I did not state specifically that during the experiment it also continued to function normally, but surely this follows. If the animal had shown irregular movements under experimental conditions, naturally I should not have published the results. I have looked carefully through my paper for any sentence which, by mistranslation, might have led Eriksson to attribute to me the statement he quotes in his footnote, but can find

* Lowndes (1933, p. 1116) claims to have discovered an entirely new filter-feeding apparatus. I have replied to his statements in a letter to " Nature " (1934, p. 329). See also Dennell (1934, p. 140).

† Not " by its back " as Lowndes erroneously states (1933, p. 1101).

none. However, I am afraid I cannot follow Eriksson's argument on this point, for, after making the reference to my demonstration of the oral current which I have just quoted he states: " Wie steht es nun bei einer Prüfung mit Cannons Auffassung vom Umfange des gegen den Mund gehenden Stromes und seiner Erklärung desselben ? Was die Annahme betrifft, dass von den Seitenkammern in die Bauchrinne Wasser ausgestossen werde, so ist dies nur eine Vermutung, den (sic) Cannon nicht einmal auf die geringste Weise als wahrscheinlich darzustellen vermochte "—that is, he refers to my experimental result as a mere conjecture.

The intermittent nature of the food stream I maintained was a result of the metachronial rhythm of the trunk limbs. " When any limb is just about to reach the end of its backstroke the limb immediately behind it commences its forward stroke. Thus, momentarily, the two limbs move in opposite direction and tend to obliterate the inter-limb space. This suddenly forces out the remaining water. It cannot pass out distally as the two limbs are in contact at the point of flexure. Some passes out laterally, while the remainder passes forwards between the basal endite and the main axis of the limb into the food groove " (1928, p. 821 and text-fig. 4, p. 811). Eriksson disputes this as he says there is no such exit from the inter-limb space (1934, p. 72). He states that where the basal endite curves downwards into the food groove (see my fig. 2, A and B, 1933, p. 273) the long setæ from its margin touch—that is, lie close against—the wall of the latter. However, the space can be seen in the photomicrograph shown in fig. 1 (e.g.), and also it is shown very clearly by Eriksson himself in his fig. 8 (p. 68).

Subsequently (1933) I showed that this exit from the inter-limb space takes the form of a definite " exit groove " which is a " channel in the wall of the food groove curving from the base of the inter-limb space towards the floor and forwards to the mouth " (p. 336).

Dr. Calman in the course of a discussion at the Zoological Society (February 6, 1934) has doubted the existence of these grooves. I can only reply that they are very definite in all the Anostraca which I have studied, and even more so in other Branchiopoda, but as I have stated (1933, p. 272) are extremely difficult to study. In only one very large form was I able to study them satisfactorily under a binocular microscope, but in all other forms I made them out with certainty from a comparison between frontal and transverse sections.

Dr. Calman also doubted whether my explanation as to how the intermittent spurts of the food current are produced is correct. He maintains that Mr. Lowndes's cinematograph films of living Chirocephalus

(Lowndes, 1933, Plates IV and V) do not show the limbs coming together and completely obliterating the inter-limb space, for there is often, if not always, a white gap between the black images of the limbs.   Now according to my hypothesis, there is no need for the inter-limb spaces to be *completely* obliterated and I have never maintained that they are.   I used the words " nearly obliterated " (1928, p. 811) and " the inter-limb space will be squeezed " (1933, p. 273), both of which statements are compatible with there being a white space between the images of the limb.

But what are these white gaps ?   Eriksson (1934, p. 58) has criticized the cinematographic method and I would go further and criticize the ordinary micro-photographic process applied to living Crustacea, of the transparent nature of *Chirocephalus*, etc.   Storch's photographs of *Sida* (1929) show the limit of what may be obtained by this method.   While they are excellent, it is usually very difficult to be certain as to the actual shape and boundaries of any special structure and this is a necessary result of the transparency and thickness of the object photographed. Now these white spaces between the dark limbs, which Dr. Calman takes to represent the inter-limb spaces, form excellent examples of structures which practically cannot be understood direct from the photographs, but must be interpreted in conjunction with the known anatomy of the limbs.   The trunk limbs of *Chirocephalus* are " Turgorextremitäten "— that is, limbs whose rigidity and functioning depends on their being distended with blood.   A frontal section of such a limb shows a relatively small section of muscle in the centre surrounded by a wide blood space. In the photographs of the living limb the dark images are produced mainly by the muscles.   Whether the front and hind surfaces of the limb separated from these muscles by transparent blood spaces will come out in the photograph depends entirely on their position relative to the direction of light.   In certain positions they may show clearly while in others they cannot possibly appear as a sharp outline and may even fail to appear.   The white spaces in Mr. Lowndes's photographs, I suggest, may represent the transparent blood spaces of the limbs. If not, then I am entitled to ask where are these blood spaces.   They cannot disappear, for if the blood is forced away from the front of the limb it will only distend the back.   It is clear that these photographs cannot settle the point.   It is possible to see so much more—and so much more clearly— by direct observation of the living animal, and as a result of such observa- tions I still maintain that as a limb ends its backstroke the limb behind commences to move forwards and this *tends to obliterate the space* between them.

The food current passes forwards to the mouth along the deep mid-ventral channel. The latter, as the name implies, is simply a gutter, but just behind the mouth it becomes a tube owing to the backward extension of the upper lip. This is illustrated in the appendix to my paper (1933, fig. 26). In this region the floor of the tube, as in the more posterior groove, is the mid-ventral ectoderm, the roof is formed by the labrum, while the walls are formed by the paragnaths and closely applied maxillules. Behind the maxillules and between them and the first trunk limbs are the minute maxillæ. It is in this region that there is a gap in the wall of the food groove (Cannon, 1933, fig. 31) through which the food stream makes its exit " and becomes caught in the backward lateral swimming streams " (Cannon, 1928, p. 813).

Eriksson (1934, p. 71) disputes this. He states that the food stream passes to the entrance of the mouth, where it comes to a standstill. But is this possible, for surely an underwater current cannot come to a stop at any point without ceasing to exist. If the food current stops anteriorly, as Eriksson suggests, then clearly it must stop all along the food groove. If any stream is to be maintained it must have a source of supply but equally it must have a sink into which it can drain. It was this obvious fact that led me to look for some mechanism whereby the food stream could be allowed its exit while at the same time the particles carried by it could be arrested in the mouth region. Without such a mechanism the food stream would be useless. Eriksson merely states that as the current comes to a standstill at the mouth entrance the particles carried by it accumulate here. But surely he is making the same mistake that he later attributes to me (see p. 456), namely, that particles can be moved independently of the currents carrying them. If the current comes to a standstill then it must cease to have any effect on the floating particles and hence they cannot accumulate.

THE FUNCTION OF THE LABRAL GLANDS

I stated (1928, p. 813) " The food particles carried on the food stream are arrested in the maxillary region by the entangling secretion of the labral glands, and the viscid mass is pushed into the mouth by the maxillules." In addition to the reasons I have just given for the necessity of some entangling mechanism I based my views largely on the function of the labral glands of a Daphnid which I described in 1922, p. 229.

Eriksson takes an opposite view. He agrees (1934, p. 86) that the function of the labral gland secretion is to entangle the food particles but maintains that this is so that they can be thrown out of the food

groove and not taken into the mouth.   It is difficult to reconcile this idea with this statement (p. 70) that the Anostraca feed on isolated and scattered particles and that it is rare that there is any accumulation in the food groove. If this is so it would appear improbable that a special structure would have been evolved to throw out surplus food on these rare occasions.

His argument (1934, p. 85), however, is that in the labrum there is a tranverse muscle running from side to side which contracts actively whenever the labrum is raised away from the floor of the food groove for the ejection of any accumulation of material, and he suggests that this muscle, by its contraction, produces an emptying of the labral glands. He assumes, as far as I can gather, that the labrum is only raised when material is to be thrown out of the food groove and deduces therefore that the secretion of the labral glands is only passed out on these occasions and hence must be for the purpose of agglutinating the food particles so that they can be thrown out *en masse*.

Now the labrum is a thin-walled finger-shaped lobe distended with blood and labral glands lie in its cavity as a bunch of scattered glands connected by a very few connective tissue cells to the ectoderm.   The levator muscles of the labrum lie in the head region and are attached to the lower side of the labrum at its anterior limit.   Any muscle such as Eriksson describes could only have the effect of supporting the labrum while it was raised by the true levator muscles.   Eriksson's views, however, must be based on some misinterpretation of his sections, for while there are paired small muscles which run obliquely from the lower to the upper surface of the labrum (figs. 4 and 5) there are no " von Seite zu Seite gehende Muskeln."   What appears as such in fig. 4 is a fold of chitin between the upper and lower halves of the bifid tip of the labrum and this is probably what Eriksson has mistaken for a muscle.   Apart from this anatomical error it must be remembered that glands, certainly skin glands in the Crustacea, are not usually emptied by muscular contraction.

I possess a collection of slides of transverse sections of *Chirocephalus*, kindly given to me by Mr. E. R. Speyer.   These were fixed in sublimate and stained in borax carmine, but in the twenty years since they were prepared have faded considerably.   In searching for specimens among them which might show food particles in the food groove, I discovered what appeared as a non-staining coagulum near the labrum.   This could only be seen with difficulty and so I re-stained the sections in an attempt to bring out this coagulum more clearly.   I found that, on staining with Mallory and not differentiating, while the tissues of the body were extremely overstained, the coagulum remained a uniform pale

slate blue.   From its distribution there can be no doubt that this coagulum
is the labral gland secretion.

Figs. 1–7, Plate 24, are photo-micrographs of sections of an adult *Chiro-cephalus* about the hinder limit of the labrum and are arranged in a series so that fig. 1 is the most posterior and fig. 7 the most anterior.*
Fig. 1 is at the level of the middle of the first inter-limb space.   Medianly the section passes through the setæ of the backwardly directed endites of the first trunk limb (*s.*1), laterally through the exites (*ex.*) and towards the bottom of the figure through the endopodites (*end.*).   The median food groove is very clearly shown and hanging down into it (that is, pointing upwards in the photograph) are the proximal parts of the basal endites of the second trunk limbs (*p.e.*2) with their filter setæ.   It will be noted that these do not lie close against the walls of the food groove but at some small distance parallel to them, as I have previously mentioned (p. 458).   Fig. 2 is slightly further forwards and cuts through the posterior side of the corm of the first trunk limb (*c.t.l.*).   It is just behind the tip of the labrum.   Fig. 3 cuts the tip of the labrum (*lab.*) and passes through the centre of the corm of the first trunk limb and through the proximal part of its basal endite.   Fig. 4 passes through the labrum and the anterior side of the first trunk limb.   The tips of the most proximal filter setæ are cut across as they project forwards on either side of the food groove.   Fig. 5 cuts through the maxillæ (*mx.*2), which can be seen as lobes on either side just above the labrum.   Fig. 6 cuts through the tips of the paragnaths (*par.*) and the hinder margin of the maxillules (*mx.*1).   In the labrum is a small portion of the scattered labral gland (*l.g.*) showing its opening to the exterior on the upper surface.   Fig. 7 passes through the paragnaths and maxillules just behind the mandibles.   As there is a slight head flexure figs. 6 and 7 are not accurately transverse to the longitudinal axis and hence the maxillules are cut obliquely.   The position of the mandibles is indicated in fig. 7.   This figure represents the entrance to the mouth.   Fig. 8, Plate 24 is from another specimen and passes through the middle of the first trunk limb.

In searching the series of sections I found, as I expected, that, while the greater number of specimens had empty food grooves, a few had the anterior part filled with a dense mass of detritus, but there were none intermediate between these two types.   This agrees with the feeding habits.   *Chirocephalus* normally feeds on isolated floating particles, but

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* The shape and relationship of the Anostracan labrum to the mouth parts and first trunk limbs are shown in my paper (1933) as an outline sketch in fig. 1 (p. 271), as a diagram in fig. 26 (p. 341), and in detail in fig. 27 (p. 343).   In the latter the tip of the labrum is raised away from the body so as to display the mouth parts.

sometimes, as I pointed out (1933, p. 279) and as Eriksson describes fully (1934, p. 7), it gathers food from the bottom while swimming dorsal side uppermost. It kicks up the detritus on the bottom by the back-stroke of its trunk limbs and then draws the concentrated disturbed matter into the median space in the usual manner. Eriksson has made a very interesting observation on this type of feeding (1934, p. 79). He maintains that the heavy spines on the margin of the endopodites are special structures for scraping up such residue, and points out that in *Polyartemia forcipata*, where they are absent, the habits are more pelagic than in other Anostraca.

Fig. 8 is from a specimen which must have kicked up a mass of food and so filled its food groove just before it was killed. It illustrates clearly the filter process. The detritus (*f.*) fills the food groove and can be seen (especially on the right-hand side of the figure) being sucked against the filter setæ.* Along the lower edge of the mass of food can be seen the coagulum of the labral gland secretion (*l.g.s.*). It is not so much entangling the food as forming a blanket covering it, and, as I shall explain from the other sections, this is, in my opinion, its function at this level of the body. In the gut can be seen not only a mass of food (*f.*) but also a large amount of the coagulum (*l.g.s.*). The evidence that this is the labral gland secretion is simply that it stains exactly the same colour both inside and outside the gut, and under high power shows the same texture. This supports my view that the entangled food is eaten and not thrown away as Eriksson maintains.

The function I attributed to the secretion was entirely that of retaining the food particles—I have never suggested that it has any digestive function. I feel I must emphasize this as Eriksson (1934, p. 86) states that it is difficult to see of what use the production of a secretion would be in front of the mouth when the œsophagus is the usual place in which it is customary to look for secretions.

Figs. 1–7 are more instructive than fig. 8 for in these there is no food to obscure the actual limits of the labral gland secretion. Fig. 1 shows a well-defined mass of secretion (*l.g.s.*) extending from one limb to the other at the level of the lower half of the basal endite. It is being sucked against the filter setæ but, as might be expected from its viscid nature, cannot pass through them. The most interesting part of this photo-graph, however, is the clear space between the coagulum and the mid-

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* Lowndes (1933, p. 1112) has put forward an entirely new view as to the mechanism whereby water is drawn in from the surroundings and filtered. If his views were correct detritus would be seen in the inter-limb space and exit groove in fig. 8, whereas it is confined to the median space.

ventral body wall where the secretion is entirely absent. This represents the forwardly directed food current (*f.c.*), and, in fact, fig. 1 can be taken as further proof, if such is necessary, of the existence of this current. Clearly, if no such current existed, there would be nothing to prevent the secretion of the labral gland extending to the floor of the food groove, but in all the sections I possess in which I have been able to stain the secretion it always shows the charcateristic appearance of fig. 1. The function of the secretion is, therefore, something more than that of merely entangling the food particles, as I originally suggested. It is secreted near the tip of the labrum and oozes round the sides and tip of the latter to form a mass under the head extending from the mandibles back to the most anterior trunk limbs. Here it is sucked against the filter setæ of the anterior limbs on their fore stroke, so that it extends completely from one side to the other of the median space. It cannot extend to the mid-ventral cuticle because of the current of water passing forwards. It therefore converts the anterior food groove into a functional tube leading straight to the mouth. In my best preparations this tube extends from the second, if not third, trunk limb. The functional significance of such a tube is obvious, for, once a particle gets into the tube, it is bound to pass to the maxillary region. Fig. 2 is more anterior than fig. 1 and shows a greater amount of secretion extending around the tip of the labrum. Just in front of this section—at about the level of the maxillary region—is the region where the food current is bound to make its exit. Fig. 3 shows this actually taking place. The continuation forwards of the functional food tube now extends round the side of the labrum into the main mass of labral gland secretion (*ex. f.c.*). The wall of this channel can be seen cut tangentially as the dark circular area in the mass of secretion shown in fig. 2. This exit channel can be followed, in the series of sections from which the photographs were taken, opening to the exterior among the irregular superficial masses of secretion. Fig. 4 shows a continuation of the exit of the food current at the left-hand side of the labrum, but instead of a clear space against the body wall there is an irregular mass of secretion (*f.l.m.*). It is in this region I consider that the food particles are entangled in the secretion. Anterior to this section (figs. 5, 6, and 7) the food groove is closed on every side (= *Atrium oris* (Eriksson, 1934, p. 74)). Above and below are the mid-ventral ectoderm (*m.v.e.*) and the labrum (*lab.*). At the sides are the maxillæ (*mx.*2, fig. 5) and immediately in front the paragnaths and maxillules (*par.* and *mx.*1, figs. 6 and 7). Anteriorly this atrium is closed by the mandibles at the actual mouth—posteriorly it opens into the food groove. Now it is into this region that the labral gland secretion is poured, and since the

atrium oris (*a.o.*, fig. 5) is closed everywhere except posteriorly the secretion must pass on the whole backwards. Hence as it escapes it meets the food stream (*f.c.*, figs. 1, 2, and 3) passing forwards from behind and any particles which are carried on this current may become entangled in the secretion.

Anteriorly to this is the region of activity of the maxillules (*mx.*1, figs. 6 and 7). These can easily be observed working together in the middle line pushing the tips of their setæ on to the mandibles. According to Eriksson (1934, p. 75), in addition, the paragnaths can be pulled laterally and thus produce a suction in the *atrium oris*, which sucks material from behind. This material, according to what I have shown above, will consist of particles carried on the food current mixed with labral gland secretion. I have not observed directly the movement of the paragnaths apart from the maxillules but, while I do not contradict Eriksson's statement, I do not think such a movement as he describes essential. The *atrium oris* will be filled with newly extruded secretion into which the food particles are forced. The movement of the maxillules is not only inwards but also backwards and forwards from the level of the maxillæ to the mandibles. As the inner surface of the maxillulary setæ are covered with forwardly directed setules, it is clear that the secretion and entangled food must be raked forwards, proportionately as the maxillules work, on to the mandibles. Here the accumulated mass is swallowed periodically by the peristaltic action of the œsophagus and I should think that the emptying action of this process, on the contents of the *atrium oris*, would be more effective than a suction action of the paragnaths.

From what I have said it is clear that, even if a particle gets as far forwards as the food tube, it does not necessarily follow that it will reach the mouth. It may slip by the labrum in the exit channel (*ex. f.c.*, fig. 3) when it will be lost. But from figs. 3 to 7 it can be seen that the labrum is in such a position that it can control the exit channel. I chose these sections for photographing because they showed one main exit stream, but in others there is more than one stream and these are much narrower than that shown in fig. 3. Clearly by pressing the labrum up against the mouth parts the ease by which the water can escape can be controlled. In addition, however, to this control I suggest that the maxillæ may play a part. From fig. 5 it can be seen that these limbs form side doors to the hind end of the *atrium oris*. By movement towards, or away from, the labrum they would tend to close or open the sides of the *atrium oris* and so hinder or allow to escape the food current and the labral secretion. I do not believe that the tips of the maxillæ are movable as there is no musculature in this minute limb, but the limb

as a whole may be moved on its base. Apart however from any active movement, the maxillæ may act as passive valves moving outwards when the pressure from behind becomes sufficiently great and so allowing a greater escape of water and closing in again when the pressure falls. I make this suggestion largely on account of the peculiar maxillæ found in another Anostracan *Branchinecta*. Miss Leak and I (Cannon, 1934, p. 34, fig. 31) figured the maxillæ of *Branchinecta gaini* in their natural position. Each consists of a plate bearing a few plumose setæ on its edge and the two plates are curved over towards the middle line so that an arch is formed over the food groove—" but one seta is always directed laterally outwards into the space between the first trunk limb and the base of the maxillule." It can be clearly seen from the figure I have quoted that, by a lateral movement of the maxillæ, or better still by a rotation, the exit of the water from the food groove could be controlled. (See also later, p. 467.)

The secretion of the labral glands therefore according to my hypothesis carries out two functions. It entangles the food particles in the *atrium oris* but also converts the anterior part of the food groove into a definite tube leading as far as the maxillary region where the exit of the food stream is probably controlled by the activity of the labrum and maxillæ. With regard to this second function, the photographs add additional evidence. At the surface of the secretion it will be noticed that in all the sections there is a layer more deeply staining than the interior mass, and moreover of a more homogeneous nature. I think there can be no doubt that this indicates a slight skin which forms at the surface as the secretion is extruded. It may be suggested that this is merely a precipitation effect of the fixative, but the photographs do not support this. In all sections posterior to the labrum the coagulated skin is always most pronounced on the surface nearest the body (figs. 1 and 2). Now, if it were produced by the precipitation effect of the fixative, it would be expected that the skin would be the same wherever it was formed. However, again it may be argued with reason that the precipitation effect on first contact with the secretion may not be so great as after the animal is half dead and the fixative has had time to penetrate to the deeper layers nearest the body. But fig. 4 goes against this for it shows the exit channel round the sides of the labrum with a very markedly coagulated wall (see also figs. 2 and 3). The photographs demonstrate in fact that the secretion in contact with the forward food current coagulates more completely than that in contact with the water in the surroundings. This suggests that the food current carries some coagulant.

Now I suggested (1933, p. 279) that the filtered food might be mixed with

a mucus-like secretion before it was blown along the food groove, for in the middle of each exit groove from the inter-limb spaces there is a conspicuous bun-shaped gland* " which most likely exudes a viscid material on the collected mass. Also on the outer face of the basal endite near its proximal margin, there is a pear-shaped gland with a conspicuous opening; whatever secretion this produces passes into the entrance of the exit groove and so must be squirted into the food groove with each spurt of the oral stream." While the last part is correct, the first suggestion is certainly incorrect because, if viscid material were poured into the food groove from all the inter-limb spaces it would certainly be present as a coagulum in the anterior region shown in fig. 1, whereas it is not. But it is possible that the secretion of these limb glands has a coagulative effect on the labral gland secretion. This would agree with the appearance shown in the photograph. In figs. 1 and 2 the secretion (*l.g.s.*) is in contact with the food stream (*f.c.*) above but not below. Hence the skin is much more marked dorsally. In fig. 3 the main mass of secretion below the labrum has never come into contact with the food stream and so the coagulated surface is not very marked, while the exit channel passing through it (*ex. f.c.*) has definite walls. In fig. 4 the mass at the sides of the labrum has again not come into contact with the food stream and by comparing its surface with the skin shown above in fig. 1, it will be seen that it has a relatively weak structure.

I have already pointed out that Eriksson considers that the labral glands only secrete when the labrum is raised. I see no reason why they should not function continuously. If the secretion remains in the *atrium oris* it must be ultimately eaten; if it passes backwards it contributes towards the wall of the functional food tube while if it oozes round the sides of the labrum it forms part of the mass underneath the head region. The specimen from which figs. 1–7 was taken had a large mass extending from side to side even in front of the mandibles. This, the photographs show, fig. 7, is covered with a very weak skin and, I imagine, gradually breaks away as the animal swims through the water.

Finally this function of forming a tube of the anterior part of the food groove may throw light on the structure of those minute limbs—the maxillæ. In all the Anostraca we have studied—and even in those Cladocera where the maxillæ persist—they always carry characteristic " soft, flexible, plumose setæ near the tip " (Cannon, 1934, p. 351).

* Lowndes (1933, p. 1110) states that staining with methylene blue brings out the presence of mucus in the food groove and of mucus glands in its wall. This is incorrect as methylene blue is not a stain for mucus glands. Eriksson (1934, p. 86) states that he could find no indication of mucus in the labral gland secretion.

These occur just where the food stream is making its exit and hence where, if my suggestion is correct, there will be a continual supply of coagulant. Now the setæ carry a large bottle brush of long setules and I suggest that such a structure is admirably adapted to retain the viscid secretion as a mass where it will coagulate—or be coagulated—and so help to close the *atrium oris* as I have previously described. At the same time the setæ are so lacking in rigidity that they could not form a permanent wall, but would easily be forced outwards on increasing water pressure from behind. In talking therefore of the action of the maxillæ (see p. 466) I consider them as structures considerably larger than their actual size owing to the adherent mass of coagulating secretion adhering to their long setules.

SUMMARY

The mechanism of the production of the anteriorly directed food current along the mid-ventral food groove of the Anostracan is again discussed. Certain statements of my views on this subject published by Eriksson (1934) are corrected.

By appropriate technique it has been possible to stain the secretion of the labral gland in sections of fixed material, figs. 1–8, Plate 24.

By studying the distribution of the labral gland secretion it has been possible to demonstrate by photographs the existence of the anteriorly directed food current which has always been denied by Storch, figs. 1–4.

The labral gland secretion makes its exit near the mouth entrance on the upper surface of the labrum, fig. 6. Part of it oozes round the sides of the labrum and forms a mass underneath the head region and mouth parts, figs. 3–7, while a part of the secretion passes backwards beyond the tip of the labrum where it is sucked against the inner surface of the anterior trunk limbs, figs. 1 and 2. Here it forms a blanket over the anteriorly directed food current converting the anterior part of the food groove into a functional tube.

The anteriorly directed food current makes its exit round the sides of the labrum at a level just behind the maxillæ through the mass of labral gland secretion, figs. 3 and 4.

Food particles may escape with this current and so be lost as food, or they may become mixed with labral gland secretion in the *atrium oris*, figs. 4–7.

The mixture of food and labral gland secretion is pushed into the mouth by the maxillules so that both food and labral gland secretion are found in the gut, fig. 8.

Cannon

Proc. Roy. Soc., B. vol. 117, *Plate* 24
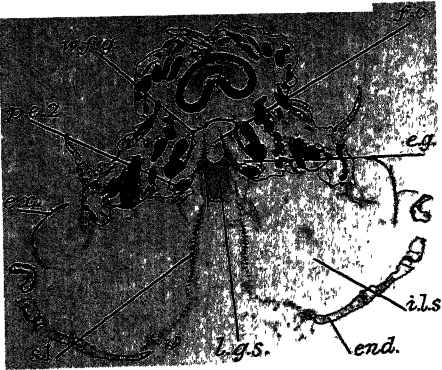
Fig. 1
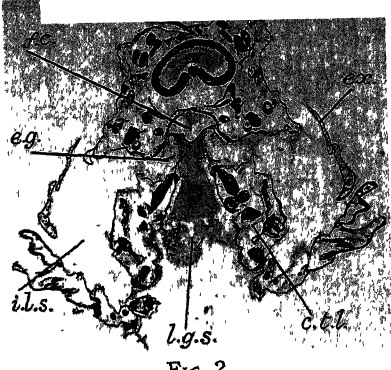
Fig. 2
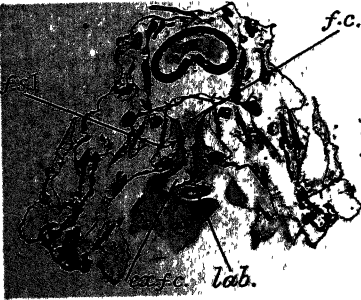
Fig. 3
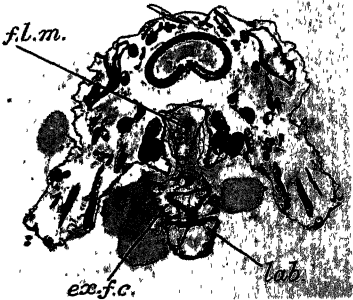
Fig. 4
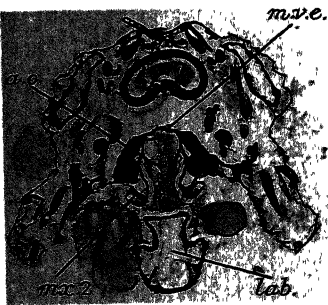
Fig. 5
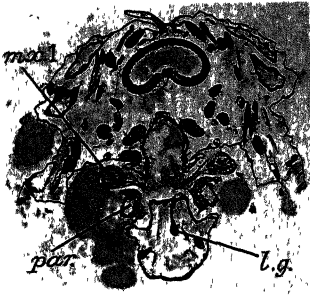
Fig. 6
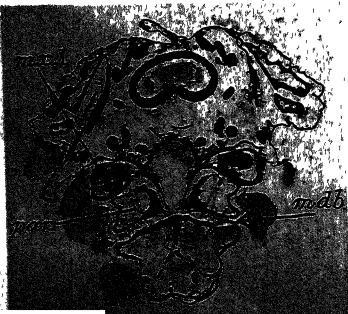
Fig. 7
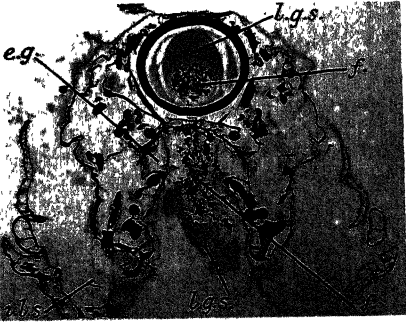
Fig. 8

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DESCRIPTION OF PLATE 24

All the photographs are of sections heavily overstained in Mallory and undifferentiated.

All are of transverse sections of *Chirocephalus diaphanus* and are arranged so that fig. 1 is the most posterior and fig. 7 the most anterior.   Fig. 8 is from another series.

ABBREVIATIONS USED

a.o., *atrium oris*.

c.t.l., corm of trunk limb.

e.g., exit groove from inter-limb space into food groove.

end., endopodite.

ex., exites.

ex. f.c., anterior food current making its exit through labral gland secretion round side of labrum.

f., food.

f.c., anteriorly directed food current.

f.l.m., mixture of labral gland secretion and food particles at entrance to *atrium oris*.

*f.s.*1, proximal filter setæ on basal endite of 1st trunk limb.

i.l.s., inter-limb space.

l.g., small portion of labral gland opening on upper surface of labrum.

l.g.s., labral gland secretion.

lab., labrum.

m.v.e., mid-ventral ectoderm.

mdb., level of mandibles.

*mx.*1, maxillule.

*mx.*2, maxilla.

par., paragnath.

*p.e.*2, proximal part of basal endite of 2nd trunk limb bearing anteriorly directed filter setæ.

*s.*1, setæ on endites of 1st trunk limb.

w.f.g., wall of food groove.

FIG. 1—At the level of the middle of the 1st inter-limb space.

FIG. 2—Cutting through the posterior side of the corm of the 1st trunk limb.

FIG. 3—Cutting through the centre of the corm of the 1st trunk limb and the tip of the labrum.

FIG. 4—Passes through the labrum and anterior side of the 1st trunk limb.

FIG. 5—Cuts through the maxillæ.

FIG. 6—Cutting through the tips of the paragnaths and the hinder margin of the maxillules.

FIG. 7—Passing just behind the mandibles, that is, at the mouth entrance.

FIG. 8—From another specimen passing through the middle of the 1st trunk limb and showing the food groove and gut filled with both food and labral gland secretion.

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591 . 55 : 595 . 789
612 . 493 : 595 . 789

The Epigamic Behaviour of *Euplœa* (*Crastia*) *core asela* (Moore) (Lepidoptera Danainæ)

By Oswald H. Latter

with a description of the Structure of the Scent Organs

By H. Eltringham, D.Sc., F.R.S.

A—The Telegamic* (Courtship) Flight of the Male *E. core asela*

By Oswald H. Latter

(Received December 13, 1934)

[Plate 25]

Fritz Müller appears to have been the first to make systematic observation and to give accurate descriptions of the scent-producing and scent-distributing organs of lepidoptera. Translations of his papers published on this subject form the Appendix to Longstaff's " Butterfly Hunting in Many Lands " (1912). In course of the first of these, *loc. cit.*, p. 612 (Müller, 1877), he writes " having therefore demonstrated . . . that the purpose of the brushes and hair-tufts in the males [of certain species] is to exhale scents, which are probably agreeable to their females and entice them to pair, I am led to infer that this is the meaning of all similar structures on the wings of male lepidoptera, not only because of the unmistakable similarity amidst such great diversity [of systematic groups],

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* The new term suggested by Dr. Eltringham in the second part of this communication is here adopted.

but even more on account of the . . . peculiarities which render them especially suitable for such a purpose. They are usually sheltered from the air . . . while at rest. Thus the scent is not diffused at the wrong time and so wasted . . . One could hardly find a more effective method of employing any odoriferous substance than that of saturating with it the hairs of a brush, and then suddenly opening them out in all directions, so as to provide an enormous surface for evaporation."

Longstaff (1912) brought together the numerous observations made by himself and Dixey on the scents of butterflies in many parts of the world. Some of these are scents pleasant to our own olfactory sense, *e.g.*, the lemon-verbena of the male Green-veined White. Dixey (1909 to 1932) has investigated the external structures which are concerned with the production and distribution of the scents, devoting special attention to the specialized scales on the wings of male Pierines; while the minute anatomy of internal organs, such as anal glands and their accessory structures, of many species have been described and figured by Eltringham (1913 to 1929 and 1934) and Freiling (1909). Structures that are almost certainly analogous occur among Neuroptera and Trichoptera; and these too have been described by Eltringham (1919, 1931, 1932) and Mosely (1919). Field observations on the use and employment of these contrivances among lepidoptera have been recorded by Carpenter (1914-1927), Champion (1930), Fyson (1930), Lamborn especially (1911 to 1921), and Lever (1931). Lamborn, on more than one occasion witnessed the application of the distributing apparatus, the anal brushes, of certain Danaines to the scent-producing brands of the wings; and at the same time " experienced a sensation as if an aromatic snuff had impinged upon the mucous membrane of my nostrils." Lever, too, describes the smell of the scent glands of certain Fijian species of *Euplœa* as " like that of burnt ginger-bread or caramelized toffee." The names of some of those who have interested themselves in this subject are given in the references. It has been assumed that when both sexes are alike equipped with scent-organs the aroma given forth serves as a deterrent against insect-eating enemies; and, on the other hand, that when possessed by one sex only it serves to attract mates. Proof of this latter assumption has long been familiar in the " assembling " of male moths attracted, often from a great distance, by a virgin female of the same species; but, except in the moths *Hepiatus hectus* and *H. humuli*, proof of the converse phenomenon, female attracted by male, has not hitherto been forthcoming; though Carpenter's observations (1914, 1927) show that the males of *Amauris psyttalea* and *Danaus chrysippus* make persistent attempts *at close quarters* to attract the females.

With a view to obtaining evidence on this point during a visit to Ceylon in the early months of 1934, I watched, at the suggestion of my old friend Professor Sir Edward B. Poulton, F.R.S., the behaviour of the Danaine butterfly *Euplœa* (*Crastia*) *core asela*, which was daily to be seen in the small garden of the bungalow in which I was staying in Colombo. The male of this species has a small narrow brand on the fore wings, and at the posterior end of the abdomen a pair of large yellow anal brushes that lie withdrawn within the body except during the courtship-flights. On these occasions they are protruded on either side, and become very conspicuous. The central part of each brush consists of a pencil of closely apposed hairs, and projects further than the peripheral portions whose hairs diverge widely from one another so that the air passes freely between them. Of some species the minute anatomy has been described by Eltringham (1913) and by Freiling (1909). On March 19, 1934, while inside the bungalow, I caught sight of two pairs of the species flying about the garden *in coitu*. I determined therefore to devote the next morning to observing the behaviour prior to actual pairing. On March 20, then, I strolled about the garden, and soon saw a male *Euplœa* flying to and fro at a height of between 3 and 4 feet above the ground over the portion of a sand-strewn path that was fully exposed to the sun. On approaching as close as I could without letting my shadow fall upon the path, I was able to note that the flight of the butterfly was of two kinds, each extending over a few yards only, but always over the sunlit patch of the path. These two modes of flight were adopted alternately, and in fairly quick succession; the first was characterized by rapid flapping of the wings, the rate of beat being decidedly faster than in ordinary straightforward flight; the other can best be termed " sailing-flight," for during these periods the wings were extended motionless; and at times the insect would hang almost stationary in the air, supported, no doubt, partly by the rising column of heated air off the sunlit patch, and partly by the very gentle N.E. breeze that came along the path after traversing the open (uncovered) passage between the north wall of the bungalow and the north boundary wall of the garden. During the sailing-flights the distal portion of the abdomen was steeply flexed in the ventral direction, so that its apex hung well below the level of the wings, and the anal brushes were fully protruded and easily visible from a distance of several yards. During flapping-flight, however, the abdomen was held horizontal, or nearly so; and though in this first observed specimen I could not satisfy myself because of the rapid fluttering of the wings, in a later specimen which was making unusually short to-and-fro flights I was able to assure myself that the

apex of the abdomen was not turned forward to touch the brands on the fore wings; and to be almost certain that the anal brushes were then completely withdrawn into their sacs. Later I found that in freshly killed and also in captive living specimens, it was impossible to obtain full extension of the brushes except by pressure with the abdomen flexed as it is during sailing-flight. It is thus most probable that during flapping-flight the brushes are retracted within the body.

After I had been watching this male for a few minutes, a female of the same species arrived on the scene, having flown there from the S.W. side of the garden, *i.e.*, against the gentle N.E. breeze, and therefore from the direction towards which the aroma emanating from the male was being wafted. The two insects at once joined in a giddy aerial dance, rose higher in the air, and flew away in company over the garden wall, and out of sight.

Before long another male started performing in exactly the same manner, and at about the same height over the same patch on the path; and almost immediately a female came up, again from the S.W. side of the garden. This male I captured for identification before they had time to whirl away. A few minutes later a third male came over the magic area of the path, and behaved exactly as its two predecessors had done, and again a female soon flew to it, again from the S.W. This male, too, I immediately netted. Within 10 minutes yet a fourth male came over the same area, and at once began the characteristic courtship-flight; and to it yet a fourth female soon came, again from the S.W. This time I did not interfere, and the two butterflies, after whirling round one another for a short time, settled on the leaves of *Ficus repens* that clothes the garden wall, and there mated. After remaining some minutes, they flew away still *in coitu*.

These observations were made between 10.30 a.m. and 1.0 p.m.; and on other days it was always in the morning that I saw males performing in this way—possibly because I was seldom in the garden in the early afternoon, possibly, too, because the shadow of a large tree fell across the magic patch of the path soon after 2.0 p.m. and for the rest of the afternoon. In the later part of the afternoon none of these butterflies ever appeared, even in the sunlit parts of the garden.

It is difficult to avoid the conclusion that in this species the aroma of the male is mainly due to a secretion received by the anal brushes from the adjacent anal glands. Dr. Eltringham's microscopic examination of the glands on the fore wing indicates that these, small though they are, are nevertheless functional. It is thus possible that the rapid vibrations of the wings during the periods of fluttering-flight are instrumental in

releasing and distributing an ingredient of the aroma that emanates from the butterfly. This conclusion received strong support from an occurrence a few·days later. I was endeavouring to get the anal brushes fully extended prior to killing a male, in the hope of being able to preserve them thus in a dried specimen; I had succeeded in securing full extension by gentle pressure of the flexed abdomen, the wings and all other parts of the insect except the distal two-thirds of the abdomen being covered by the palms and fingers of my hands, when a female flew towards me and hovered for a few seconds at less than a foot from my hands above the captive male, which I then released to fly away uninjured. In this case the wings had been covered by my hands for a minute or so before the female arrived; and moreover their dorsal (upper) surfaces were closely apposed to one another, thus minimizing the opportunity of escape of any aroma produced by the glands. Freiling (1909) considers that it is from the glandular apparatus connected with the brushes that the aromatic substance is produced; and his drawings of the highly magnified hairs show minute pores opening in the axils (to borrow a botanical term) of short pointed projections that jut out from the surface of the delicate distal portion of the hairs; and here and there in the axils, and blocking the pores, are indicated particles of a substance which he regards as the secretion from which the aroma emanates. The axillary cups may well serve as temporary depots for the odoriferous secretion. My own observations appear to substantiate Freiling's view.

I did not detect any aroma proceeding from any of the several specimens that I handled; nor did I perceive any fine dust-like particles such as have been described as liberated by the brushes of some other species (Eltringham, 1915). Longstaff, however, whose sense of smell would appear to have been exceptionally acute, speaks of this same species having a scent that suggested to him " rancid oil, or old lamps," and quotes Mr. Scott's comparison of it to acetylene. But inasmuch as Longstaff sometimes detected the scent in specimens of both sexes, it seems certain that it was a defensive rather than a sexually attractive quality.

Be this as it may, the observations here recorded appear to establish beyond question that in *Euplœa core asela* the anal brushes of the male do, as has long been assumed, distribute an aroma which is attractive or stimulating to the female. They furthermore prove that, in this and probably many other allied species, the aroma actually causes the female to seek the male. Nor is it irrational *ex uno discere omnes*, and to conclude that similar structures in other male insects have a like function, as Fritz Müller suggested more than half a century ago.

It is remarkable that the courtship-flights which I witnessed, not only on

March 20, but also on other subsequent days, were invariably executed at the same spot in the garden, namely, over the sunlit patch of the path; they never extended over the lawn which reaches right up to the path, nor over the parts of the path that were shaded, to the west by a large tree, to the east by the bungalow. Why did the courtship-flights occur here and here only ?

Of others who have noted these flights, Mrs. D. R. Fyson (1930) alone describes the spot over which they were performed. She speaks of a male *Euplœa core* flying more or less in a circle over an almost bare patch not more than 3 yards in diameter; occasionally it made a hovering [sailing] flight across, and then the tail pointed downwards well clear of the wings, and the two brushes were protruded. There was a bank of succulents on one side and a large tree trunk on the other side of the patch, so that the space was confined; it was partially shaded, but the foliage being thin (March 10, 1930) the sun shone through. She makes no mention of the direction or of the strength of such wind as there may have been at the time.

On March 13, 1930, Mrs. Fyson saw another male *Euplœa core* behaving in the same manner over a part of her drive which was shaded (to what extent is not stated) by tall trees, and bordered by a low clipped hedge on either side. This was a more open space than that of March 10, and the circular flight of the insect was less regular. It is noteworthy, however, that the area over which the courtship-flight was being executed was confined on two sides by compact hedges. The butterfly does not appear to have been satisfied with this spot, for after performing for 2 minutes from the moment when it was first observed, it flew away.

There is, then, a considerable resemblance between the spots at which Mrs. Fyson and I respectively witnessed these courtship-flights; all three spots were somewhat confined, hers as just stated, mine by the 6-feet high north wall of the garden and by the bungalow itself—the path, 13 feet wide, continues eastward between the garden wall and the north wall of the bungalow, whose north-west corner is about 10 feet from the favoured spot; two also were exposed to the sun, the first of hers moderately, mine strongly. I suggest that this latter feature is the determining factor, and the former perhaps a not unimportant subsidiary.

A bare patch of sandy soil exposed to direct sunshine, especially in the tropics, becomes extremely hot; a continuous column of heated air rises from it, and, if dead calm prevails, cooler air from all sides will continuously flow in to take its place, provided there be no obstacle on any side. My belief is that the rising column of hot air furnishes the support for the stationary wings of the insect during the sailing-flights, and that

obstacles which to a greater or less degree confine the heated area cause the inflow of air to be mainly from one direction, so that the aroma is wafted along a fairly definite course by the resultant of the vertical rising component and the horizontal one due to the inflow of air being from one direction only. The effect will thus be more potent than if the aroma were simply carried upwards by the heated column, and then dispersed at random in many directions.

In the instances that I have here recorded the gentle N.E. breeze certainly appeared to have this effect, for every one of the four females attracted came to the spot against wind. It is noteworthy that the courtship-flights were executed comparatively close to the ground, that is, where the up-current of air is most marked. In all probability the rising column of heated air is the stimulus which reflexly initiates the courtship-flight, and rapidly volatilizes the material to which the aroma is due.

[*Note added in proof*, *April* 27, 1935—Since the above was written, my daughter (Mrs. H. E. Newnham) in a letter dated February 13, 1935, writes: " I have seen quite a lot of your butterflies the last few weeks flirting over that sandy patch at the end of the lawn." This observation seems to me very interesting and to support my idea that the particular conditions—heated soil, sheltered position, etc.—are required to stimulate the butterfly to execute its courtship-flight.]

B—On the Structure of the Scent Organs in the Male *E. core asela*

By H. ELTRINGHAM, D.Sc., F.R.S.

My friend, Professor Sir Edward Poulton, F.R.S., has handed me a copy of Mr. O. H. Latter's account of his observations on the courtship of the butterfly *Euplœa core asela*, and the accompanying use of the scent-brushes of the male. We are deeply indebted to Mr. G. M. Henry, of the Colombo Museum, for a supply of material of the species, carefully preserved and packed, from which the sections here described have been made.

As will be learned from Mr. Latter's paper and the references therewith, special scent-organs in male insects are of frequent occurrence and have long been known. The organs in *Euplœa* are not of an unusual character, but Mr. Latter's observations are entirely new, and show that the scent-apparatus is used, at least in this species, *to attract the female from a distance. This function*, which I venture to term *telegamic*, has not

hitherto been recognized except perhaps in moths of the genus *Hepialus*, in which the action occurs over only very short distances. The scents produced by male insects have been supposed to be rather of an aphrodisiac character, coming into play only when contact, or at least proximity has been attained. Many female moths produce a directive scent, invariably, so far as is at present known, imperceptible to the human sense. Male moths will fly from great distances (experimentally for more than a mile) in response to its stimulus. Such females as have been microscopically examined do not possess special glands for the secretion of these directive odours. The whole hypodermal layer underlying the terminal segments of the abdomen is modified into a secreting epithelium, and the scent appears to be diffused by osmosis. Even among day-flying moths, sight does not appear to be an important factor in the communication of the sexes. In the majority of the butterflies, however, sight seems to be of first importance, though the butterfly's eye is not more highly developed than is that of most moths. Whilst scent organs of considerable complexity are of continual occurrence in moths, they tend to be of a simpler character in butterflies, being usually in the form of special wing-scales which secrete a scent, frequently perceptible to the human sense. Nothing corresponding with the directive odours of female moths has so far been observed in butterflies, and of those species in which scent organs have been found, they occur only in the males, except when the odours produced are defensive, when they may be found in both sexes, as in the genus *Heliconius*. A high development of male scent-organs is, however, found in the Danaine butterflies, and especially in the genus *Amauris*, the species of which have active scent-glands in the hind wings and extrusible abdominal brushes. Müller, in 1877, suggested a possible correlation between the brushes and the wing-glands, but it was not until 34 years later that the actual application of the brush to the gland was observed by Lamborn in Africa. A peculiar feature in some species is that a dust is formed by the breaking up of specially secreted fine hairs which pulverize into small particles. This dust may be formed either in the brush, as in *Amauris psyttalea*, or in the wing-glands, as in those species in which the gland is in the form of a pocket.

In *A. psyttalea* the dust material is secreted in comparatively enormous quantities, about two-thirds of the brush-bag being occupied by it, and Carpenter has observed the male hovering over the female and evidently dusting her with the scented powder. In species where the wing-glands are absent, as in *Hestia*, or very poorly developed, as in *Euplœa*, the brushes themselves may be glandular, and it is with a brush

of this type that we have to deal in *Euplœa core asela*. Nevertheless the wing-gland, which in *Euplœa* is on the front wing, instead of on the hind wing as in the Danainæ, small though it is, is active, and its relation to the brushes is not clearly understood. Mr. Latter did not observe any contact between the brushes and the wing-glands, and at first sight it would seem to require rather an acrobatic feat on the part of the insect to touch the front wings with the brushes. Mr. T. Bainbrigge-Fletcher, however, informs me that he has frequently seen the insect applying its brushes to the fore wings, though after such a lapse of time he is unable to recall the precise position adopted in so doing. The brush hairs project approximately at right-angles to the long axis of the organ, and one could imagine the abdomen being either turned upwards and for-wards, after the manner of a scorpion, or bent sideways and forwards.

Whilst it will be seen from the description of the histological structure of the wing-gland that there is evidently a secretion of some kind, it must be very small in comparison with that of the brushes. Mr. Bainbrigge-Fletcher tells me that he had always supposed that the insect was anointing its wings with the secretion from the brushes, and that it had not occurred to him that the wing-gland was functional. The wings would thus be the distributive mechanism, but Mr. Latter's observations indicate that the brushes really serve this purpose, as, from the analogy of other species, we should expect. How then are we to explain the action of the wing-glands ?

In a South American butterfly, *Opsiphanes cassiœ*, there are two erectile brushes, and both are in the wing instead of in the abdomen. One is non-glandular and evidently purely distributive. It is so placed as to be in contact, when the wings are closed, with a large and actively secreting gland on the side of the abdomen. The other brush cannot in any conceivable position come into contact with the abdominal gland, but it lies, when at rest, on a gland of its own, situated in the wing-membrane. When describing this apparatus (Eltringham, 1929), I made the suggestion that the wing-gland and the abdominal gland might conceivably produce two different volatile substances which, when mixed, produced, by some " nascent " chemical action, the characteristic perfume of the species. I can adduce no experimental support for the theory, but if such an action be possible, the same idea might be applied to the combined action of the brushes and wing-glands of *Euplœa*. Given an unlimited supply of fresh material, bred from pupæ, and so in a fresh and active state, proof of, or at least support for, this theory should not be impossible of achievement, provided the odour produced is perceptible to the human sense. It remains only to describe the structure of the organs in *Euplœa core asela*.

Fig. 1, Plate 25, shows a longitudinal section of one of the abdominal brushes under a low power. The brush in its membranous sac is about 7 mm long. The hairs, H, arise from rather elongated sockets, and they are much more numerous at the base of the sac than in the more distal parts. Thus when everted and the brush expanded, the tuft will be denser at its extremity. The eversion of the brush is caused by fluid pressure from within, and its withdrawl secured by muscles attached to the base of the sac. Some of these muscles remain in the section at M. It will be noted, even at a low magnification, that the substance of the sac or brush-bag is much thicker than a mere trichogenic membrane. In the majority of my sections the cells of the brush-bag are highly vacuolated, the nuclei small or vestigial, and the cell-contents scanty and ill defined. This is characteristic of some insect gland-cells which, through the activity of their abundant secretion, tend to break down altogether. The metabolic balance is upset, a fact of no great importance when the accomplishment of the purpose of the secretion is likely to be quickly attained. A single hair-socket with its glandular cells in a less exhausted state is shown at fig. 2, Plate 25, much enlarged. There is an expansion, E, at the root of the hair, bounded by a layer of cytoplasm having a more or less columnar structure, beneath which, faintly indicated, there is a funnel-shaped formation F. The remainder of the cell, or more probably, cells, shows no special structure when stained with hæmatoxylin. Four nuclei are visible in this preparation, but the number varies. The diameter of the hairs is about 0·016 mm. For some distance from their origin they are comparatively smooth, with a very faint longitudinal striation, and an irregular series of markings, which seem to be fibres in the interior and slight irregularities on the surface. A small part of a hair in this stage is shown in surface view at fig. 3, Plate 25. More distally each hair develops an elaborate " sculpture," which I have endeavoured to indicate at fig. 4, Plate 25. Carefully examined under the oil-immersion objective, the scale-like projections present a faintly striated surface, and there are occasional dots which might be, but cannot certainly be interpreted as, pores. The presence or absence of pores in these hairs has never been certainly determined. Freiling (1909) describes and illustrates such pores, but I am still uncertain of their existence. It would seem possible to decide the point by the use of the Chambers' micro-injection apparatus, a costly equipment I regret I do not possess. A transverse section of one of the hairs is shown at fig. 5, Plate 25. In this example there is a small central lumen, but usually the interior seems to be entirely filled with an irregular and very delicate tissue, a common feature of insect hairs of this kind. I have found no traces of any

secretion in or upon the hairs, but any such secretion may be soluble in one or more of the fluids used for fixation and sectioning. It would not seem necessary that there should be visible pores in the hair-walls. Some insect gland-tissues, as in *Heliconius* and the scent-scales of many butterflies, seem to excrete by a process of diffusion. At the base of the brush-bag the lining membrane is comparatively smooth, but more distally it becomes papillate, as in fig. 6, Plate 25, which shows a small piece of this membrane. The whole surface is covered with very minute projections, and there are larger button-like excrescences irregularly scattered over it. There are no visible pores in the membrane. Excreting membranes associated with scent-brushes, and perforated by easily visible pores, are not unknown, a good example being the membrane in a moth, *Lithosia griseola*, described by me in a paper which I hope may shortly be published.

Fig. 7, Plate 25, is a diagram of the fore wing. The minute gland or scent patch is indicated at B. If this part of the wing be cleared and stained, the appearance in surface view is as at fig. 8, Plate 25. I have left four of the covering scales in position. They are rather smaller than the surrounding wing-scales. Small gland-cells can be seen in the space between the wing-membranes. A section of the glandular area is shown at fig. 9, Plate 25. The cells exhibit a faint structure which could probably be more clearly elucidated by special cytological methods. Some are evidently in communication with the scale-sockets, as at C, but there are also intermediate cells, as at D, which may contribute to the secretion by diffusion through the delicate septa S, S, by which the cells are separated.

On the whole, the brush of *E. core asela* is very similar to that of *Trepsichrois mulciber*, described by me (Eltringham, 1915) and probably functions in a similar manner. Glandular brushes of a like form are found in the genus *Hestia*, though here there is no wing-gland, but there are four abdominal brushes instead of two. Two of the four are glandular, and the other two distributive. I have described them in the same paper.

SUMMARY

Observations on the behaviour of the male butterfly *Euplœa core asela* Moore, show that it habitually flies in places where the natural air currents diffuse a scent emanating from a pair of abdominal brushes.

The effect of this proceeding is to attract females from a distance, the action being described as *telegamic*.

A somewhat similar action has been observed in certain moths of the genus *Hepialus*, but not apparently effective over any considerable distance.

The brushes are glandular and evidently produce a secretion, but there are also small but active glands in the fore wings of the insect, and there is some evidence that the brushes are occasionally applied to these glands.

Illustrations and descriptions of the histology of the organs concerned are given, and the suggestion is made that the mixing of the secretions of the brush and wing glands may possibly produce the characteristic odour by nascent chemical action.

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DESCRIPTION OF PLATE 25

Fig. 1—Longitudinal section of base of abdominal brush in *Euplœa core asela* ♂.
   H, hairs, M, muscle.

Fig. 2—A single hair-socket with glandular cells, more highly magnified.   E, expansion
   of hair-base; F, funnel-shaped striation in cytoplasm.

Fig. 3—Surface view of basal part of a single hair.

Fig. 4—Ditto of more distal part of same.

Fig. 5—Transverse section of distal part of a hair.

Fig. 6—Fragment of lining membrane of brush-bag, showing nodular structure.

Fig. 7—Diagram of the fore wing of the butterfly.   B, the wing-gland.

Fig. 8—Surface view of part of wing-gland, with four of the covering scales remaining
   attached.

Fig. 9—Section of part of wing-gland.   C, one of the gland-cells communicating
   with a scale-socket; D, an intermediate gland-cell; S, S, septa separating the
   scales.

   Actual measurements—

Total length of brush in retracted state, 7 mm.	Length of wing-gland, 3·5 mm.
Average diameter of a hair, 0·016 mm.	Thickness of wing in fig. 9, 0·04 mm.
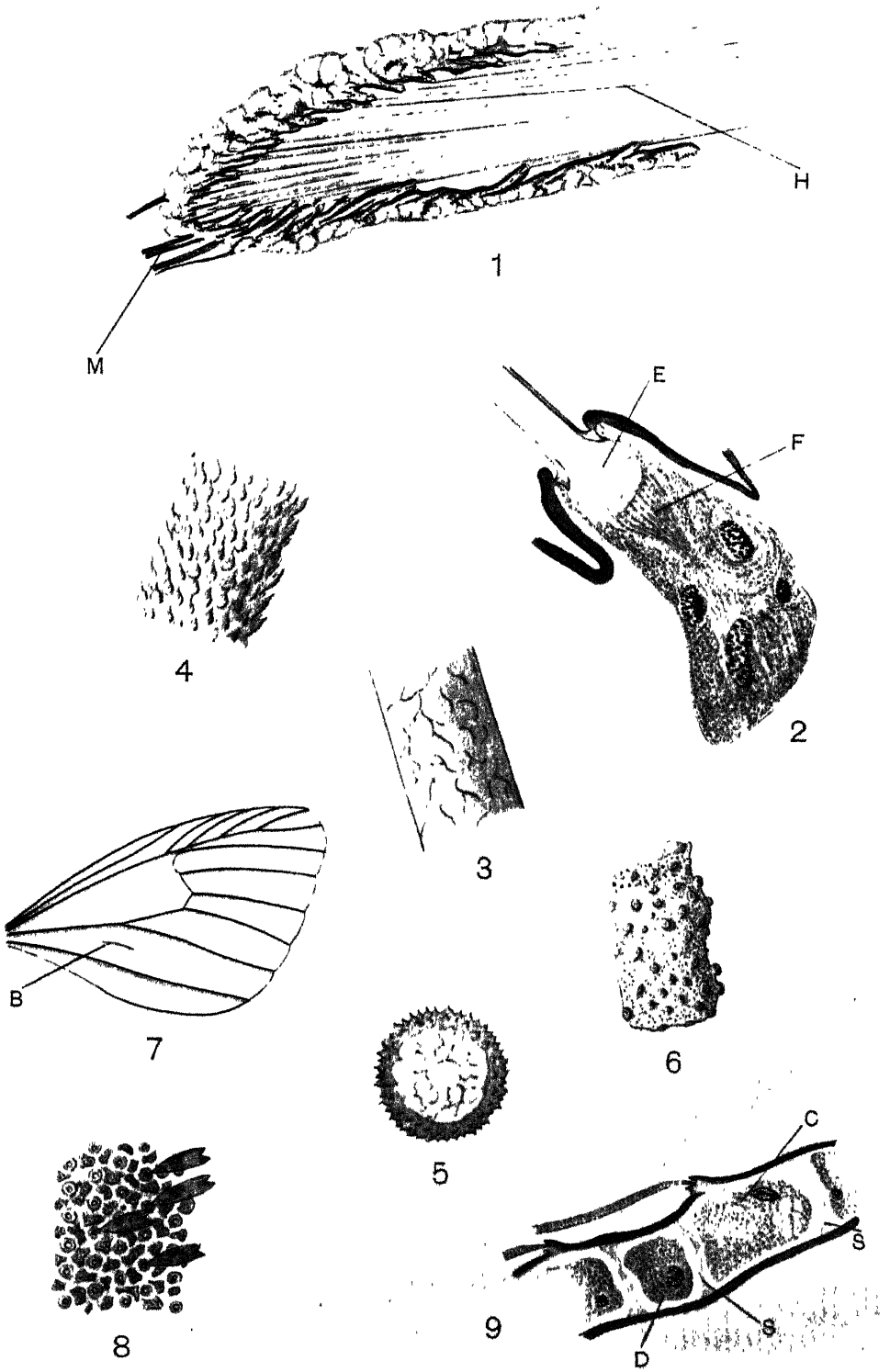
The Respiration of Barley Plants
I—Methods for the Determination of Carbohydrates in Leaves

By EDMUND W. YEMM, Christopher Welch Biological Scholar (1931)

(From the Department of Botany, Oxford)

There is much evidence to show that under normal circumstances the respiration of higher plants involves the oxidative breakdown of hexose carbohydrate. During leaf starvation under carefully controlled external conditions,* a gradual exhaustion of the carbohydrate substrate is, therefore, one of the important internal changes likely to affect the rate of carbon-dioxide production. The researches to be described here, and in a subsequent paper, were planned in order to test this possibility; an attempt has been made to discover the relation between the concentration of readily available carbohydrate and the rate of carbon-dioxide production in starving leaves.

The chief experimental difficulty involved was that of making accurate determinations of the various hexose sources present in the leaf, and methods whereby this could be done were tested extensively. Since the validity of many of the conclusions reached depends primarily on the accuracy of the analytical methods, it was considered important that an account of these should be included here. It is with this phase of the problem that the present paper is concerned.

In a survey of the carbohydrates of the Gramineæ, de Cugnac (1931) found that sucrose, glucose, and fructose were the chief soluble sugars present in barley leaves. No glucoside could be detected, while the fructosan, present in other vegetative parts appeared to be absent from the leaf lamina. Maltose was not found, and under normal circumstances starch is only present in very small quantities, usually at the base of the leaf. Thus the analytical treatment aimed at a determination of glucose, fructose, sucrose, and starch, while methods for the detection of maltose were also developed, as the presence of this sugar would seriously affect

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* A full description of the starvation conditions will be given later by Dr. W. O. James.

the other estimations. The possibility of fructosans occurring in barley leaf extracts has been kept in mind; the reactions of these compounds, described by de Cugnac (1931), make it clear that pure fructose will be formed on hydrolysis, if they are present.

Widdowson (1931) has described a method for the estimation of small quantities of mixed reducing sugars by a combination of modified Hagedorn-Jensen and iodimetric methods. These two methods have been used extensively in this investigation. Each of them was first tested with pure sugar solutions and artificial mixtures of those sugars likely to occur in leaf extracts. Such modification and extension of the procedure, as was necessary in the analysis of leaf extracts, is described in a later section, together with the method of starch determination.

I—ANALYSIS OF PURE SUGAR SOLUTIONS

1—MODIFIED HAGEDORN-JENSEN METHOD

The Hagedorn-Jensen method, as modified by Hanes (1929), was used for the determination of total reducing sugar, including both aldose and ketose. The experimental procedure was exactly similar to that described by him. Titrations, never exceeding 4 in number, were carried out concurrently, so that about 8 minutes elapsed between the formation of free iodine and the final titration. In this time no significant loss of iodine could be detected, and cooling with ice was unnecessary.

Using a similar glucose preparation to that of Hanes (1929) (B.D.H. analytical reagent), and desiccating for 48 hours over calcium chloride, reducing values in close agreement with his were recorded. The values observed by Widdowson (1931) are slightly higher than these, a difference attributed to the fact that, after desiccation described above, the preparation still contained approximately 2% of moisture. With a view to testing this, the specific rotation of the glucose preparation, after desiccation over calcium chloride, was estimated and gave a value $[\alpha]_{20}^{D}$ 51·3, compared with the accurate value $[\alpha]_{20}^{D}$ 52·75. If the preparation was further desiccated by heating to 100° C for 3 hours, reducing values very close to those of Widdowson (1931) were found.

The method has similarly been standardized by Widdowson (1931) for pure desiccated fructose and maltose, both alone and in presence of glucose. While it has not been possible to confirm all these results in the present work, the use of invert sugar solutions and pure B.D.H. maltose has shown close agreement with the results given, and, in conse-

TABLE I—REDUCING VALUES OF GLUCOSE BY THE HAGEDORN-JENSEN
METHOD

Glucose mg	Equivalent cc 0·01 N thiosulphate	Equivalent cc 0·01 N thiosulphate (Widdowson)
3·14	9·66	9·68
3·11	9·62	9·60
2·95	9·10	9·08
2·00	6·03	6·04
0·99	2·99	3·00

quence, these standardization values have been used for the conversion
of titration figures to milligrams of reducing sugars.

2—IODIMETRIC ESTIMATION OF ALDOSE

In the use of this method it has not been possible to follow the exact
procedure of Widdowson (1931). The change has necessitated con-
siderable restandardization of the method under new experimental
conditions.

Sodium hydroxide was the alkali used, as it gave more rapid oxidation
and yielded quite consistent results in presence of sucrose and fructose.
A controlled temperature of 20° C was adopted for the oxidation, since
it was impossible to maintain a steady temperature lower than this under
the laboratory conditions. The following solutions were used to meet
the experimental requirements:—

A—Approximately N/40 solution of iodine; B—2% sodium hydroxide;
C—3% sulphuric acid; D—standardized approximately N/75
sodium thiosulphate.

The procedure for determination of a sugar solution was as follows:—
5 cc of the solution were pipetted into a thin-walled boiling tube
6 inches × ¾ inch and 5 cc of iodine solution added, followed by 1 cc of
alkali. This latter manipulation was carried out as rapidly as possible
to prevent loss of iodine. The tube was then tightly corked, and im-
mersed in a water bath at 20° C for sufficient time for the complete
oxidation of aldose to occur. After this time 1 cc of acid was added,
and the residual iodine titrated against the standard thiosulphate. A
distilled water blank, made up by substituting 5 cc of distilled water for
the sugar solution, was treated in an exactly similar way. Thus by
difference from the blank the cc N/100 thiosulphate equivalent to the
sugar could be determined. These determinations were always carried

out in duplicate, the agreement generally being within 0·02 cc N/75 thiosulphate.

With the reagents described the range of the iodimetric method is almost the same as that of the Hagedorn-Jensen. In the former method the titration differences are considerably reduced, but this is to some extent compensated by an increased accuracy, owing to the simpler manipulation.

The time necessary for complete oxidation was determined with pure solutions of glucose at both high and low concentrations (2·46–0·49 mg per 5 cc). It was always found that no further detectable oxidation occurred after the first 15 minutes, and this was shown to be true also for pure maltose solutions.

The mean of a large number of determinations carried out with this oxidation period gave the following standardization figures for glucose and maltose:—

1 mg glucose equivalent to 1·12 cc N/100 thiosulphate.
1 mg maltose equivalent to 0·59 cc N/100 thiosulphate.

These values agree closely with those determined by Widdowson (1931), who used a temperature of 1° C, and an oxidation period of 2 hours.

In order to determine the correction necessary for the slow oxidation of ketoses and sucrose, the oxidation of glucose in presence of these sugars was examined under the standard conditions. The solutions containing glucose and fructose were prepared by mixing pure glucose and invert sugar in varying proportions.

The results of a series of such experiments are summarized in Table II; the figures of column 5 represent the titration values of the different solutions; in column 6 these values are calculated to cc of 0·01 N thiosulphate equivalent to 1 mg of glucose in presence of the other sugars.

From the figures of column 6 the following mean values were calculated:—

Glucose in presence of fructose:
1 mg glucose ≡ ʹ 1·15 cc 0·01 N thiosulphate.

Glucose in presence of fructose and sucrose:
1 mg glucose ≡ ʹ 1·17 cc of 0·01 N thiosulphate.

At the extremes of concentration of sucrose and fructose it is apparent that deviation from this mean value occurred. The range of concentration investigated roughly corresponds to that likely to arise in dealing

with plant extracts, and it appears that error, from this source alone, could not exceed 2% of the glucose value. In consideration of this, a more elaborate correction was considered unnecessary; the values given on p. 486 have been used for the iodimetric determination of glucose in presence of fructose and sucrose.

The iodine oxidation of a solution containing known quantities of glucose, fructose, maltose, and sucrose was examined. It was found that, if the corrected glucose value (1 mg $\equiv^t$ 1·17 cc) was applied, the maltose could be considered as unaffected by the presence of the other sugars (*i.e.*, 1 mg $\equiv^t$ 0·59 cc 0·01 N thiosulphate).

TABLE II—OXIDATION OF GLUCOSE IN PRESENCE OF FRUCTOSE AND SUCROSE

Exp No	mg glucose (5 cc)	mg fructose (5 cc)	mg sucrose (5 cc)	cc 0·01 N thiosulphate equivalent to sugar present	cc 0·01 thiosulphate equivalent to 1 mg glucose
1	2·51	0·37	0	2·84	1·13
2	1·63	0·74	0	1·86	1·14
3	2·01	0·88	0	2·30	1·14
4	0·52	0·52	0	0·60	1·13
5	1·43	1·43	0	1·66	1·16
6	1·66	1·66	0	1·96	1·17
7	1·46	1·46	6·14	1·73	1·18
8	1·46	1·46	3·07	1·72	1·17
9	1·46	1·46	1·53	1·73	1·18
10	1·46	1·46	0·77	1·71	1·17
11	2·01	0·88	6·60	2·37	1·17
12	2·28	0·09	1·60	2·65	1·16

3—THE DETERMINATION OF SUCROSE IN PRESENCE OF GLUCOSE, FRUCTOSE AND MALTOSE

By means of a range of pure sucrose solutions (B.D.H. analytical reagent) containing from 0·5–7·5 mg per 5 cc, it was shown that this sugar had no effect on the modified Hagedorn-Jensen method, either alone or in presence of glucose and fructose. The largest effect, which was observed at higher concentrations, was equivalent to 0·04 cc of 0·01 N thiosulphate, and this was barely outside the limit of experimental error. Thus, the determination of the increase in glucose and fructose on complete hydrolysis gave a method of sucrose estimation by the Hagedorn-Jensen method. Similarly the iodimetric method could

be used with the corrections described above. In this case the increase in glucose only is estimated, so that this value must be doubled to give the total hexose formed from sucrose hydrolysis.

A variety of hydrolysing agents have been used by past workers, a primary consideration being that none of the other sugars present should be affected. Citric acid 10% was tried in some preliminary experiments under the conditions adopted by Davis and Daish (1913). This was found to give consistently high values, as compared with other methods, probably owing to the effect of citric acid itself on the reagents. The following hydrolysis method was finally adopted, after Lehmann (1931).

20 cc of sugar solution were pipetted into a large boiling tube and heated to 70° C in a water bath. The solution was acidified with 2 cc of 27·5% sulphuric acid, and kept at 70° C for 10 minutes. After this time it was cooled rapidly in running water, and then sufficient 15% alkali added to neutralize to phenolphthalein. The actual amount of alkali needed was determined on a duplicate sample, so that no indicator was added to the solution to be analysed. The neutralized solution was then made up to a convenient volume, and 5 cc aliquots were used for Hagedorn-Jensen and iodimetric determinations. Trial of this hydrolysis procedure with weighed quantities of pure desiccated sucrose gave values in close agreement with the mean of the appropriate values for glucose and fructose, as determined by Widdowson (1931).

TABLE III—REDUCING VALUES OF INVERT SUGAR SOLUTIONS

mg sucrose added 5 cc	mg invert sugar 5 cc	0·01 N thiosulphate equivalent cc	$\frac{G + F}{2}$ cc 0·01 thiosulphate (Widdowson)
1·98	2·08	6·09	6·14
0·99	1·04	3·10	3·10
0·49	0·52	1·49	1·51

In numerous experiments the iodimetric and Hagedorn-Jensen methods were compared on the same invert sugar solution. It was always found that the glucose value agreed very closely with half the total sugar, estimated by the Hagedorn-Jensen titration.

The sulphuric acid hydrolysis method could be applied equally well to the determination of sucrose in solutions containing glucose and fructose, since the reducing values of these sugars was shown to be un-

affected by the treatment. For maltose a very slight increase could be detected, owing to hydrolysis.

TABLE IV—INCREASE IN REDUCING VALUE OF MALTOSE, ON HYDROLYSIS WITH 2·5% SULPHURIC ACID, AT 70° C FOR 10 MINUTES

Exp No	Before hydrolysis cc 0·01 N thiosulphate	After hydrolysis cc 0·01 N thiosulphate	Increase %
1	5·40	5·44	0·8
2	4·98	5·03	1·0
3	1·46	1·47	0·6
4	1·08	1·08	0

It can be seen that the increase was very small in all cases, and no appreciable error in sucrose estimation could arise from this source.

Hydrolysis by means of an invertase preparation was also applied successfully to the separate estimation of sucrose. 20 cc of the sugar solution were incubated with 5 cc of 0·5% invertase, 5 cc of M/5 acetate buffer, p_H 5·2, and a few drops of toluol. A blank determination, in which 20 cc of distilled water were substituted for the sugar solution, was necessary, since the enzyme preparation had a slight effect on the different methods. It was found that a period of 36 hours at 32° C was sufficient to ensure complete hydrolysis. After incubation the solution was boiled, filtered, and then made up to a convenient volume, 5 cc aliquots being used for iodimetric and Hagedorn-Jensen estimations.

A comparison of the invertase and acid hydrolyses methods on the same sucrose solution consistently gave close agreement, whether the glucose, or the glucose and fructose, were determined.

TABLE V—COMPARISON OF ACID AND INVERTASE HYDROLYSIS

Exp No	Acid hydrolysis		Invertase hydrolysis	
	Hagedorn-Jensen Glucose + fructose mg	Iodimetric Glucose mg	Hagedorn-Jensen Glucose + fructose mg	Iodimetric glucose mg
1	2·79	1·39	2·80	1·40
2	1·40	0·69	1·40	0·69
3	1·00	0·51	1·01	0·50

The same B.D.H. invertase preparation was used throughout the work, and it was shown by experiment that it had no effect on maltose solutions.

In consequence, the method could be used with confidence in presence of this sugar.

The advantage of using both the Hagedorn-Jensen and iodimetric methods for the estimation of sucrose is that the detection of fructosans in plant extracts is made possible. De Cugnac (1931) has shown that these compounds closely resemble sucrose in their susceptibility to hydrolysis by weak acids. Thus, under conditions involved in sucrose determination, fructosans will be hydrolysed, giving rise to pure fructose. If this actually occurs, the increase in total reducing sugars, measured by the Hagedorn-Jensen, will be greater than twice the increase in glucose only, as determined by the iodimetric method.

4—Determination of Maltose in Presence of Glucose, Fructose and Sucrose

From the preceding discussion of the methods, it is clear that in a mixture containing glucose, fructose, and maltose, provided a separate estimation of maltose is made, glucose and fructose can be determined from the Hagedorn-Jensen and iodimetric values. Except for the selective fermentation used by Davis and Daish (1913), the only method available for separate maltose determination depends upon the increase in reducing power on hydrolysis to glucose. The complete hydrolysis by means of dilute acids has been shown to involve considerable destruction of fructose, and, in consequence, the taka diastase method was used.

When maltose is hydrolysed Lehmann (1931) observed a 25% increase in reducing power, as measured by the Hagedorn-Jensen method. This increase is relatively small, and the experimental error in estimating small quantities of maltose would be considerable. If the iodimetric method is used, the complete hydrolysis of maltose to glucose should cause theoretically a 100% increase in reducing power, and the likelihood of large errors arising is diminished. On this account the iodimetric method has been adopted for the determination of the increase on taka diastase hydrolysis.

A special " undiluted " taka diastase preparation was kindly supplied by Parke Davis & Co.; its action on pure maltose was tested, the procedure being similar to that in carrying out invertase hydrolysis.

The increase was shown to be slightly less than the theoretical 100% value, probably due to the establishment of a glucose ⇌ maltose equilibrium. In presence of glucose and fructose the increase tended to

become more irregular—variation from 90–97% was recorded—a number of determinations giving a mean of 94%.

TABLE VI—TAKA DIASTASE HYDROLYSIS OF PURE MALTOSE SOLUTION

Exp No	Before hydrolysis cc 0·01 N thiosulphate	After hydrolysis cc 0·01 N thiosulphate	Increase %
1	1·35	2·66	97
2	1·62	3·18	96
3	1·37	2·68	96

When the mixture contained sucrose the separate estimation of maltose was further complicated. The taka diastase preparation was shown to contain active invertase, and thus, on incubation in presence of sucrose, .an increase in reducing power occurred, due both to sucrose inversion and to maltose hydrolysis. This difficulty was overcome by the use of the B.D.H. invertase preparation. The action of both the preparations was tested on the same pure sucrose solutions, and it was found that no significant difference could be detected by the iodimetric method.

TABLE VII—HYDROLYSIS OF PURE SUCROSE SOLUTION BY INVERTASE
AND TAKA DIASTASE PREPARATION

Exp No	Invertase preparation cc 0·01 N thiosulphate	Taka diastase preparation cc 0·01 N thiosulphate
1	2·29	2·28
2	2·20	2·21
3	1·62	1·62
4	1·14	1·14
5	1·10	1·10

As previously shown, the invertase preparation had no hydrolytic effect on pure maltose. This fact made it possible to make a separate determination of the increase in iodine oxidation, due to sucrose inversion only. The difference between this and the value after hydrolysis by the taka diastase preparation gave an estimate of the increase from maltose hydrolysis. The application of this method was tested on a number of solutions containing the two sugars in varying proportions.

From the results it is apparent that the increase, due to maltose hydrolysis, varies from 93–99% of the initial value. Trial with various enzyme concentrations, and longer incubation periods failed to eliminate this variation, and, in view of this, the average of the above and other deter-

TABLE VIII—HYDROLYSIS OF MALTOSE IN PRESENCE OF SUCROSE
BY TAKA DIASTASE AND INVERTASE PREPARATION

Exp No	Initial maltose cc 0·01 N thiosulphate	After invertase cc 0·01 N thiosulphate	After taka diastase cc 0·01 N thiosulphate	Difference cc 0·01 N thiosulphate	Increase % maltose
1	0	1·62	1·62	0	0
2	0·35	1·55	1·89	0·34	97
3	0·70	1·50	2·15	0·65	93
4	1·03	1·42	2·44	1·02	99
5	1·37	1·35	2·68	1·33	98

minations has been used, *i.e.*, increase on taka diastase hydrolysis is equivalent to 95% of the initial maltose value.

5—TOTAL PROCEDURE FOR THE ANALYSIS OF SOLUTIONS CONTAINING GLUCOSE, FRUCTOSE, MALTOSE, AND SUCROSE

At this point it is convenient to summarize the methods which have been developed for the analysis of mixed solutions of pure sugars. These methods have been used on numerous occasions to the determination of such solutions, and have yielded consistently accurate results.

i. Initial determination—

5 cc aliquots $\begin{cases} \text{Hagedorn-Jensen} \equiv \text{' glucose + fructose +} \\ \quad \text{maltose} \\ \text{iodimetric} \equiv \text{' glucose + maltose.} \end{cases}$

ii. After 2·5% sulphuric acid hydrolysis—

5 cc aliquots $\begin{cases} \text{H.J.} \equiv \text{' glucose + fructose + maltose + invert} \\ \quad \text{sugar} \\ I_2 \equiv \text{' glucose + fructose + } \dfrac{\text{invert sugar}}{2} . \end{cases}$

(Sucrose from ii–i.)

iii. After invertase hydrolysis—

5 cc aliquots $\begin{cases} \text{H.J.} \equiv \text{' glucose + fructose + maltose + invert} \\ \quad \text{sugar} \\ I_2 \equiv \text{' glucose + maltose + } \dfrac{\text{invert sugar}}{2} . \end{cases}$

(Sucrose from iii–i.)

iv. After taka diastase hydrolysis—

5 cc .... Iodimetric $\equiv$ ^{t} glucose $+ \dfrac{\text{invert sugar}}{2}$

(Initial maltose from 100/95 × (iv–iii).)

The methods were tested with solution made up from weighed quantities of the various sugars, pure glucose, maltose, and sucrose samples being used as previously. The fructose was a Harrington " pure " preparation, which was tested and gave 97% of Widdowson's (1931) reducing value for carefully purified fructose. The results of a typical analysis are given in Table IX.

TABLE IX—ANALYSIS OF PURE SUGAR MIXTURE

	Added gm	Found gm	Found %
Glucose	0·2007	0·2020	100·6
Fructose	0·1210	0·1140	94
Maltose	0·1209	0·1200	99
Sucrose........................	0·6138	0·6062	99
Total........................	1·0564	1·0422	99

The relatively large deviation observed in the fructose value must in part be attributed to the impurity of the preparation used.

II—ANALYSIS OF PLANT EXTRACTS

6—METHOD OF EXTRACTION

The soluble sugars were extracted from the leaf by means of 80% alcohol, an advantage of this method being that the material could be fixed and stored without danger of changes occurring, as was shown by Lehmann (1931). The usual precautions were taken to ensure rapid killing, and to prevent sucrose hydrolysis—the leaves were cut into small strips, and dropped into boiling alcohol, to which a little sodium carbonate had been added.

In dealing with leaf material of 5–10 gm fresh weight, 200 cc of alcohol were used for the killing and first extraction. Two further extractions, with 75 cc of boiling 80% alcohol for 4 hours each, were found to be sufficient to remove all the sugar. After such treatment the leaf material was colourless, and further extraction gave no detectable sugar.

The complete alcoholic extract was distilled at a pressure of 3–4 cm of mercury. It was found to be of advantage to bubble a slow stream

of air through the liquid during distillation, thereby preventing excessive frothing, and ensuring rapid distillation—350–400 cc could be distilled off in 45–60 minutes.

The extraction and distillation methods were tested to discover whether any appreciable change or loss of sugar occurred. Weighed quantities of glucose, fructose, maltose, and sucrose were dissolved in 80% alcohol, and boiled for 4 hours under a reflux condenser. The alcohol was then distilled off, and the sugars redissolved in distilled water for estimation.

TABLE X—SUGAR RECOVERY AFTER EXTRACTION AND DISTILLATION TREATMENT

	Added gm	Found gm	Recovery %
Glucose	0·0776	0·0768	99
Fructose	0·1052	0·1072	102
Maltose	0·1132	0·1080	95
Sucrose...................	0·1120	0·1126	100·4
Total.................	0·4080	0·4046	99

The results showed that under the conditions of extraction and distillation little change or loss of sugar occurs—the largest observed was 5% for maltose, while the recovery of total sugar was within 1% of the amount added.

7—CLEARING AND DECOLOURIZATION OF PLANT EXTRACTS

After the distillation of alcohol from plant extracts, the residue contained considerable quantities of chlorophyll and other colloidal material in addition to the soluble sugar. Careful extractions of the residue with warm distilled water gave dark green colloidal solutions. In the past, basic or neutral lead acetate have been widely used for clearing such solutions; this method was tested and applied in some preliminary experiments. No loss of sugar could be detected from pure solutions when they were treated with neutral lead acetate, and the lead finally removed as phosphate. The leaf extract, after treatment with sufficient lead acetate to precipitate all the colloidal impurity, was filtered, and then the slight excess removed. The resulting clear solution was pale yellow in colour, and a number of analyses were carried out on such solutions. In all tests it was found that the value for glucose, as determined by the iodimetric method, exceeded the value for glucose and fructose by the Hagedorn-Jensen method.

TABLE XI—HAGEDORN-JENSEN AND IODIMETRIC DETERMINATIONS
OF PLANT EXTRACTS

Exp No	Glucose iodimetric mg	Glucose + fructose Hagedorn-Jensen mg
1	82·0	55·2
2	85·0	68·0
3	86·8	60·4
4	70·6	44·8
5	123·0	81·0
6	109·0	90·0
7	82·4	23·2

The iodimetric method, as observed by Mcleod and Robison (1929) and, later, Widdowson (1931), is very sensitive to impurities. It seemed clear, therefore, that the discrepancy was due to this, the iodimetric method being more sensitive than the Hagedorn-Jensen. That the pale yellow colouring matter not eliminated by lead acetate might be responsible was suspected, and the use of B.D.H. decolourizing charcoal, as a means of removing it, was investigated. A considerable loss of sugar from pure solutions was observed on prolonged boiling with charcoal. However, if charcoal in quantities not exceeding 0·25 gm per 100 cc was boiled for not longer than 5 minutes the loss of sugar was very small.

TABLE XII—LOSS OF PURE SUGARS ON BOILING FOR 5 MINUTES
WITH 0·25 GM OF CHARCOAL

	Before charcoal treatment mg	After charcoal treatment mg	Loss %
Glucose	1·04	1·04	0
Fructose	1·07	1·06	0·9
Sucrose..........	2·07	2·06	0·5
Maltose	2·63	2·59	1·5

When leaf extracts, which had been treated with both lead acetate and decolourizing charcoal, were analysed, the discrepancy between the iodimetric and Hagedorn-Jensen methods persisted, although it was reduced. In the absence of a method of purification by which the impurities could be removed without affecting the sugar, investigation was made of means for removing all the sugar from the solution by fermentation with yeast. If this could be done quantitatively then the effect of the unfermentable non-sugar impurity could be determined by Hagedorn-Jensen and iodi-

metric estimations. The fermentation of solutions, which had been treated with lead acetate, necessitated the very careful removal of lead by hydrogen sulphide. A number of different reagents were tried in an attempt to eliminate this lengthy procedure, and aluminium hydroxide suspension proved to be a satisfactory substitute for lead acetate. The method finally adopted for clearing and decolourizing plant extracts was as follows.

The residue, after alcohol distillation was taken up in 50 cc of warm distilled water, with three washings, and enough of a 20% suspension of aluminium hydroxide was added to give complete precipitation of colloidal impurities. After 10 minutes' standing with frequent shaking the solution was filtered—forced filtration making it possible to carry this out in 45–60 minutes with two thorough washings of the precipitate. The clear yellow solution was then boiled for 5 minutes with 0·2 gm of decolourizing charcoal, filtered, and the charcoal washed with three successive quantities of boiling water.

Trial with pure solutions of glucose, fructose, sucrose, and maltose showed that no appreciable loss occurred, provided the aluminium hydroxide and charcoal were thoroughly washed after filtration. Similarly it was found that the fermentation of the sugars by yeast was not affected.

8—DETERMINATION OF THE UNFERMENTABLE NON-SUGAR RESIDUE

The following methods for removal of the sugars by fermentation were tested:—(1) fermentation by zymin preparations; (2) inoculation of sterilized sugar solution by a little pure brewer's yeast; (3) rapid fermentation by baker's yeast. The first of these methods proved to be entirely irregular and unsatisfactory, while the second one had serious disadvantages owing to the necessity for autoclaving and incubation for 30 days to ensure complete removal of the sugar.

For the rapid fermentation by baker's yeast, which was used extensively, the exact procedure was as follows:—

20 cc of the sugar solution and 5 cc of a 10% yeast suspension were mixed in a large boiling-tube, and placed in a water bath at a temperature of 35° C. A steady stream of air was drawn through the mixture to prevent the yeast from settling, and to remove the products of fermentation. After a period long enough for the complete removal of the sugar, the yeast was precipitated by the addition of a little 20% aluminium hydroxide and then filtered off. The filtrate containing the unfermentable impurity was then diluted to a convenient volume, 5 cc aliquots being used for Hagedorn-Jensen and iodimetric determinations.

Experiment showed that it was necessary to carry out a blank determination, substituting 20 cc of distilled water for the sugar solution, since the addition of the yeast itself had a slight effect on the different estimations.

The above method was tested with pure sugar solutions, made up to contain not more than 100 mg of sugar per 100 cc. In every test the residual sugar after 3 hours' fermentation never exceeded 1% of the initial amount present. This was true of glucose, fructose, maltose, and sucrose, both in pure solution and in mixtures of these sugars.

A series of experiments were planned to investigate the effect of the unfermentable residue from plant extracts on the iodimetric and Hagedorn-Jensen methods. The thiosulphate equivalent of the unfermentable residue was converted to milligrams of sugar, and the subtraction of this from the value before fermentation gave the actual milligrams of sugar present in the solution originally. Experiments of this type showed consistently that the impurities had a much greater effect on the iodimetric than on the Hagedorn-Jensen method.

TABLE XIII—DETERMINATION OF UNFERMENTABLE IMPURITIES IN PLANT EXTRACTS

Exp No	Initial determination mg sugar		After fermentation $\equiv^t$ mg sugar		Difference fermentable sugar mg	
	Hagedorn-Jensen	Iodi-metric	Hagedorn-Jensen	Iodi-metric	Hagedorn-Jensen	Iodi-metric
1	74	80	25	62·5	49	17·5
2	75	78	37·5	55	37·5	23
3	95	99	27	35	68	64
4	100	134	32	77	68	57
5	90	109	27	49	63	60
6	101	129·5	35	53	76	76·5

The fermentation was carried out under various conditions, and the unfermentable residue from 20 cc aliquots of the same extract always showed close agreement. The addition of pure sugars to the extract also had no appreciable effect on the determination of impurity by this method. Therefore, in all analyses of leaf extracts it was necessary to determine the effect of the impurities on the Hagedorn-Jensen and iodimetric estimations, and, after the application of the correction, these methods were used to determine glucose and fructose.

The demonstration of the serious effect of impurities on the analyses made it important to discover whether their presence affected the estimation of sucrose and maltose. This was done by subjecting the unfermentable residue from a number of different extracts to treatment similar to that involved in these estimations. Incubation with invertase and taka diastase preparations showed consistently that the Hagedorn-Jensen and iodimetric values of the impurity were unaffected. With 2·5% acid hydrolysis the results were irregular—sometimes quite a marked increase was observed.

TABLE XIV—EFFECT OF ACID HYDROLYSIS ON IMPURITIES

Exp No	Before hydrolysis $\equiv^t$ mg sugar		After hydrolysis $\equiv^t$ mg sugar	
	Hagedorn-Jensen	Iodimetric	Hagedorn-Jensen	Iodimetric
1	31	39	78	50
2	32	95	30	98
3	25	97·5	37	96
4	29	47	67	55
5	27	49	29	52

Therefore, in applying the methods previously developed for pure sugar solutions, the following additional determinations have been necessary in analysing leaf extracts:—

(v) Determination of the effect of the unfermentable residue on the Hagedorn-Jensen and iodimetric methods.

(vi) Similar determination after acid hydrolysis.

9—ADDITION OF PURE SUGARS TO PLANT EXTRACTS

The accuracy of the methods was tested by the addition of known quantities of pure sugars to extracts, and estimations of the amount recovered. An alcoholic extract of barley leaves was divided into two aliquots of 100 cc, to one of which were added 20 cc of a solution containing weighed quantities of sugar. Both of the solutions were evaporated by distillation, and the fermentable sugars estimated. Two such experiments were carried out, and the results are summarized in Table XV.

In both experiments a slight loss of sugar was observed, amounting to 2·1% of the total sugar in the experiment I and 4·4% in experiment II. It appears probable that this loss occurred in the clearing of the solution, a little sugar being carried down with the colloidal material. Since the

precipitation technique has been kept constant throughout, errors from this source must be small in determining relative concentration in similar samples.

TABLE XV

Experiment I

	Extract only mg	Extract + sugar mg	Difference found mg	Sugar added mg	Recovered %
Glucose	15·2	68·4	53·2	54	98·5
Fructose	5·2	36·4	31·2	31·6	99
Sucrose	63·6	237·2	173·6	176·4	98
Maltose	0	45·2	45·2	48·0	94
Total	84	387·2	303·2	310	98

Experiment II

	Extract only mg	Extract + sugar mg	Difference found mg	Sugar added mg	Recovered %
Glucose	25·0	46·0	21·0	19·9	105
Fructose	2·5	17·0	14·5	15·6	93
Sucrose	47·4	133·0	85·6	90·2	95
Maltose	0	0	0	0	—
Total	74·9	196·0	121·1	125·7	95·6

10—ANALYSIS OF STARVED LEAVES

With progressive starvation of the leaves certain well-marked changes were observed in the extracts obtained for analysis. While the leaf remained green, the extract, after clearing and decolourizing treatment, was practically colourless, but, on yellowing of the leaf, the colour became more conspicuous. At the same time an increase in the unfermentable impurity occurred to such an extent that the non-sugar fraction formed the larger part of the initial iodimetric and Hagedorn-Jensen values.*

While the low sugar concentration, characteristic of yellowed leaf material, inevitably increases the experimental error in determination, nevertheless it was observed consistently that in the coloured extracts the iodimetric value was greater than the Hagedorn-Jensen. The

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* It is improbable that the increase in the unfermentable residue was due to the production of reducing substances by the yeast.

explanation of this is almost certainly that the yeast takes up a little colouring matter, which could not be removed by prolonged washing. Hence it is probable that the determination of fermentable sugar in such solutions is too high: the iodimetric method being more sensitive is more eriously affected.

TABLE XVI—ANALYSIS OF YELLOW LEAVES

Exp No	Initial determination mg sugar		After fermentation mg sugar		Difference fermentable sugar mg	
	Hagedorn-Jensen	Iodi-metric	Hagedorn-Jensen	Iodi-metric	Hagedorn-Jensen	Iodi-metric
1	37·6	118·4	27·6	95·6	10·6	22·8
2	24·4	62·4	16·0	50·0	8·4	12·4
3	27·4	96·0	22·4	88·6	5·0	7·4
4	60	135	45	106	15	29

In view of the possibility that changes in the unfermentable residue, concurrent with starvation, might seriously affect the determination of glucose and fructose in the early stages, a further method of analysis was developed.

11—ESTIMATION OF FRUCTOSE BY ACID· DESTRUCTION

The method depends upon the relative instability of fructose on heating in acid solution, and has been standardized by Lehmann (1931). Preliminary work with pure sugar solutions gave results differing from his, probably owing to some undetected difference in procedure.

Hydrochloric acid, standardized against anhydrous sodium carbonate, was added to the sugar solution to give a final concentration of exactly 5%. The mixture was then heated under a reflux condenser in a boiling-water bath for a standard period of 3 hours. The acid was neutralized with 15% alkali, and the solution made up to a known volume for estimation of the residual sugar by the Hagedorn-Jensen method.

Previous work had indicated that in barley leaves fructose/glucose ratios of less than one were usually encountered, therefore the method was tested over a range of pure solutions containing known quantities of glucose and fructose. For this purpose B.D.H. analytical reagents glucose and sucrose were used, since on acid treatment the latter sugar is immediately hydrolysed to glucose and fructose.

From these figures a standardization graph was constructed to show the relation between the ratio of fructose to glucose added, and that found by

acid treatment. The graph could then be used to estimate glucose and fructose in unknown solutions, since by determining the ratio of destroyed to undestroyed sugar this can then be converted back to the ratio originally present.

Maltose was never detected in barley leaf extracts, so that its hydrolysis under these conditions did not affect the determination, while sucrose can be considered as a mixture of equal quantities of glucose and fructose. It was necessary to carry out a separate estimation of the effect of the acid treatment on the unfermentable impurities, as any change in these would affect the ratio.

TABLE XVII—ACID DESTRUCTION OF FRUCTOSE IN PRESENCE OF GLUCOSE

Sugar added			Sugar found after treatment		
Glucose mg	Fructose mg	F/G ratio	Glucose mg	Fructose mg	F/G ratio
1·35	1·35	1·00	1·60	1·10	0·69
0·34	0·34	1·00	0·40	0·28	0·70
1·65	1·02	0·62	1·79	0·88	0·49
1·16	0·54	0·47	1·23	0·47	0·38
1·90	0·70	0·37	1·98	0·62	0·31
1·90	0·70	0·37	1·99	0·61	0·30
1·92	0·68	0·35	2·00	0·60	0·30
1·60	0·36	0·23	1·62	0·34	0·21
2·04	0·18	0·09	2·05	0·17	0·08
0·62	0	0	0·62	0	0
2·48	0	0	2·45	0·03	0·01

The application of this method to the analysis of glucose and fructose in leaf extracts was not extensive. It was developed with a view to

TABLE XVIII—COMPARISON OF ACID DESTRUCTION METHOD WITH COMBINED HAGEDORN-JENSEN AND IODIMETRIC METHOD

Extract No	Combined Hagedorn-Jensen iodimetric		Acid destruction method	
	Total glucose mg	Total fructose mg	Total glucose mg	Total fructose mg
1	148	157	146	159
2	145	127	142	130
3	108	47·7	110	45

testing the accuracy of glucose and fructose determination by the combined Hagedorn-Jensen and iodimetric methods—both were used in the analyses of the same extracts, and a direct comparison of the results could be made.

Extract No. 3 was made from yellow leaves, which had been isolated from the plant under starvation conditions for 60 hours. The agreement between the different methods indicated that, up to this stage at least, confidence can be placed in the estimations of glucose and fructose.

III—STARCH DETERMINATION

The colourless plant residue, after all the soluble sugars had been extracted by alcohol, was used for starch determination. The material was dried in an electric oven at 100° C, and then ground as finely as possible. To this powder 50 cc of water were added, and the mixture boiled on a water bath for 1 hour to gelatinize the starch. 10 cc of a 1·5% taka diastase solution together with 10 cc of a M/5 acetate buffer solution, p_H 5·2, and a few drops of toluol were added. A distilled water blank was made up similarly, except for the omission of the leaf material, and both of the solutions were incubated at 32° C for 36 hours. After this time the digest was boiled and filtered, being washed thoroughly three times with hot water to remove the last traces of sugar. A little aluminium hydroxide was added to clear the solution, and then it was made up to a known volume for estimation of reducing sugar.

Iodimetric and Hagedorn-Jensen determinations have been used by Widdowson to estimate the glucose and maltose formed from starch hydrolysis; this method has been adopted in this work. It was found that with the special taka diastase available pure starch was almost completely hydrolysed to glucose. This was shown by the agreement between the iodimetric and Hagedorn-Jensen methods, if the appropriate glucose factors were used.

TABLE XIX—HYDROLYSIS PRODUCTS OF PURE STARCH.

Exp No	Glucose formed mg	
	Hagedorn-Jensen	Iodimetric
1	152	147·5
2	201	197

The slightly lower value by the iodimetric method indicated that a little maltose remained, but this was so small as to be within the limits of experimental error.

In the analysis of leaves the same difficulty, due to the presence of impurities, was encountered—in many tests the iodimetric value of the filtrate was greater than the Hagedorn-Jensen. It was overcome by making separate determination of the impurity by means of the rapid fermentation method already described. Provided this was done, the two methods agreed closely, as had been observed with pure starch preparations.

TABLE XX—STARCH DETERMINATION IN BARLEY LEAVES

Extract No	Initial determination mg glucose		After fermentation $\equiv$ ^{t} mg glucose		mg fermentable sugar mg glucose	
	Hagedorn-Jenson	Iodi-metric	Hagedorn-Jensen	Iodi-metric	Hagedorn-Jensen	Iodi-metric
1	147	173	73	99	75	74
2	125	168	75	116	50	52
3	136	164	71	103	65	61
4	109	150	60	104	49	46

In this way starch, including dextrins, has been estimated as glucose formed on taka diastase hydrolysis, taking the mean of Hagedorn-Jensen and iodimetric values.

A number of experiments was carried out in which the whole process of gelatinization and incubation with taka diastase was repeated a second time. No further production of fermentable sugar could be detected in any such experiment.

CONCLUSION AND SUMMARY

Methods have been developed for the analysis of glucose, fructose, maltose, and sucrose by a combination of iodimetric and modified Hagedorn-Jensen estimations. With pure sugars and artificial mixtures of sugar consistently accurate results were recorded with the procedure adopted.

In the analysis of leaf extracts difficulty was caused by the presence of non-sugar impurities, which affected each of the above estimations to a different extent. No adequate means of purification could be found; the alternative method of removing all the sugars by rapid yeast fermentation was adopted. In this way a separate determination of the effect of the impurity could be made. The disadvantage of the method

was that not only the sugar but also some of the impurity might be removed or new reducing substances added by the yeast. Evidence has been presented to show that this is likely to be appreciable only in the highly coloured extracts characteristic of starved and yellow leaves.

The determination of starch was carried out by taka diastase hydrolysis and estimation of the reducing sugar formed. Here again a correction for the effect of non-sugar impurities was necessary.

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The Respiration of Barley Plants

II—Carbohydrate Concentration, and Carbon Dioxide Production in Starving Leaves

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1—INTRODUCTION

The analytical methods described in the previous paper were applied to the specific problem of leaf respiration in a series of experiments extending over two years. Four separate experiments of the same type were carried out in 1932, and a further one in the following season. Experience gained in the first year led to a slight modification of the experimental procedure and to the development of gasometric methods, by means of which it was possible to measure the respiratory quotient of starving leaves. Consequently, in later experiments, it was possible to compare the results of direct sugar analysis with meaurements of the quotient, each method providing a clue to the nature of the oxidations

underlying carbon dioxide production. The standard Pettenkoffer method was used, when measurement of carbon dioxide output only was required, while the total gaseous exchange was determined by means of the Haldane gas analysis apparatus (Haldane, 1912). A full description of the gasometric methods will be given elsewhere.

2—EXPERIMENTAL DETAILS AND RESULTS

In each season barley plants of the Plumage Archer variety were grown on the same experimental plot at Islip with full mineral treatment. A more carefully controlled method of culture was impracticable, owing to the need for large numbers of leaves; in different experiments from 300 to 700 leaves were required, all isolated from the growing plants at the same time. Immediately after detachment they were divided up into separate samples of 45 to 50 leaves, and the carbon dioxide production of each sample was followed during starvation under controlled conditions. After various intervals of time, samples were analysed to find the concentration of the various hexose sources. In this way the inter-related processes of sugar exhaustion and carbon dioxide production could be followed.

(a) Sampling Method

Godwin and Bishop (1927) have shown that the metabolic age of the leaf has a marked effect on its respiration under starvation conditions. Hence, it was necessary to standardize this feature as far as possible. The third leaf inserted on the axis, counting the rolled apical one as the first, has been used predominantly, since it was found that this leaf could be considered as mature and functional. In the later stages of growth, when the emergence of new leaves had ceased, the leaf inserted immediately below the one subtending the developing ear was selected.

The size of the respiration chambers made it necessary to cut strips of leaf approximately 15 cm long; this was done on the plot, the upper half of the leaf being used in most experiments. Diseased leaves were avoided, and the extreme tip was usually removed, as it frequently showed signs of premature yellowing. In all experiments detachment from the plant was carried out between 3.30 p.m. and 5.30 p.m., so as to ensure a high initial concentration of carbohydrate. The leaf-strips were removed to the laboratory in a vasculum lined with damp cloth, and here they were thoroughly mixed and divided at random into samples of 45–50 strips each. The fresh weight of the samples was recorded as rapidly as possible, and then they were sealed into the light-proof chambers and immersed in the constant-temperature water bath.

2 o 2

(b) *Measurement of Carbon Dioxide Production and Removal of*
Samples for Analysis

In all the experiments to be described here the carbon dioxide pro-
duction record does not start until 6 hours after the isolation of the leaf
from the plant. This represents the time necessary for manipulation,
and attainment of the steady temperature of the water bath. At the
start of every experiment, the air current was drawn through a bye-pass
for 1½ hours before any measurement of carbon dioxide production
was made.

Periodic back titration of the residual baryta water against standard
acid gave a continuous record of the rate of carbon-dioxide production
of each sample up to the time of its removal for analysis. In the experi-
ments of the season 1932 11-hour periods were used, but the rapidity
with which changes occurred led to a more detailed study of the early
stages. Therefore in the next year 6-hourly back titrations were used
up to the 54th hour after isolation. A further difference was the lowering
of the steady temperature of 25° C to 22·5° C in the later experiments.
The individual features of the separate experiments are summarized
below.

Experiment I—Leaf-strips isolated on July 2, 1932. The growing
plants were about 2 feet in height, and no ears were visible. 500 leaf-
strips (upper 15 cm including the tip) were cut and divided into 12 separate
random samples. Carbon dioxide production was measured over
11-hour periods during starvation at 25° C. Samples were fixed for
analysis, initially, after 12 hours, and subsequently at 24-hour intervals.

Experiment II—Leaf-strips isolated on August 5, 1932. The developing
ears were visible and in some the yellowing of older leaves was obvious.
Approximately 250 leaf-strips (upper half with the tip removed) were cut
and divided into five random samples. Carbon dioxide production
was measured over 11-hour periods during starvation at 25° C, samples
being fixed for analysis initially, and after 12, 36, 132, and 240 hours.

Experiment III—This was carried out concurrently with experiment
II, the lower half of the leaf being used. 360 leaf-strips were isolated
and divided into nine random samples. Carbon dioxide output was
measured as before, and samples were removed for analysis initially,
and after 12, 36, 60, 84, 108, 132, 156, and 204 hours.

Experiment IV—Leaf-strips were detached on August 12, 1932. The
growing ears had completely emerged and practically all the lower

leaves were yellow. Six-hourly determinations of carbon dioxide output were made during starvation at 25° C and samples were removed for analysis initially, and after 6, 12, and 36 hours.

Experiment V—The leaves were detached on July 2, 1933, when the young ears were just emerging. Approximately 700 leaves were cut (upper half with the tip removed) and divided into 13 random samples. Six-hourly determinations of carbon dioxide production were made up
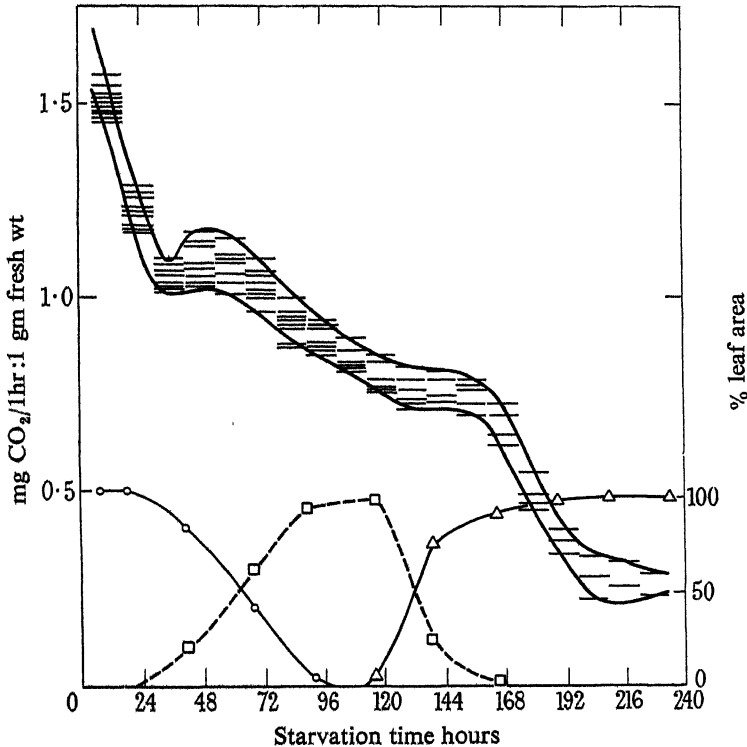
FIG. 1—CO_2 production of barley leaf samples during starvation at 25° C. Yellowing and browning of the leaves represented as % area curves. ○ Green; □ yellow; △ brown; ═ mean CO_2

to 48 hours, and afterwards at 12-hourly intervals during starvation at 22·5° C. Samples were removed for analysis initially, and after 6, 12, 24, 30, 36, 42, 60, 84, and 108 hours. A random sample of 45 leaves was used for concurrent measurements of the respiratory quotient, experiment VII.

Examples of the carbon dioxide production records are summarized in figs. 1 and 2. The rate of production has been calculated to mg CO_2 per hour, per 1 gm fresh weight, the value for the separate samples being

represented by horizontal lines. Limiting curves have been drawn to include all the different estimations.

The variation between individual samples, as shown by these records, is relatively small, the deviation from the arithmetic mean rarely exceeding 5%, except at the lower residual values after 140 hours' starvation. This has been found consistently in all experiments of this type; the " probable
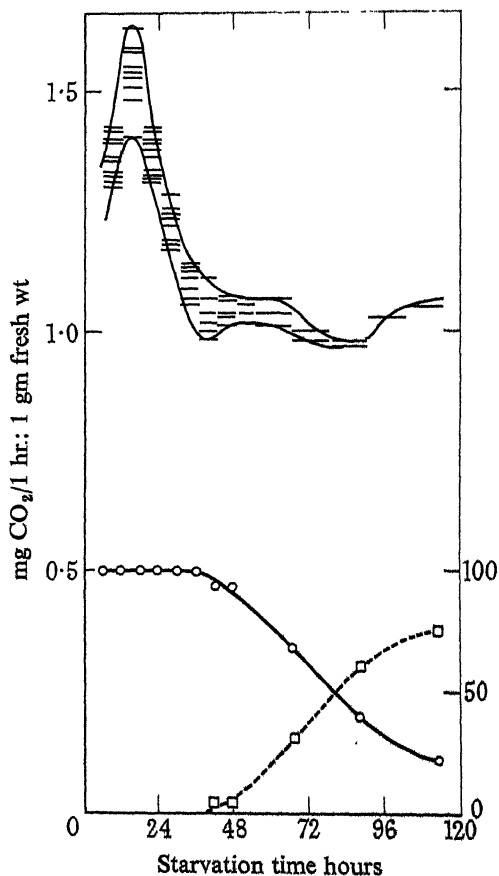
FIG. 2—CO_2 production of barley leaf samples during starvation at 22·5° C. Yellowing represented as % area curves. ○ Green; □ yellow; ═ mean CO_2

error " calculated from the usual formula gave values of $\pm 3\cdot6\%$, $\pm 3\cdot9\%$, $\pm 3\cdot0\%$ for periods showing a maximum scatter of the individual estimations.

In these and other experiments a large number of such records have been obtained, and the drift of the carbon dioxide production rate with starvation time has always shown certain consistent features. These are summarized below.

1. Immediately after isolation, a rise in the rate of carbon dioxide production occurs, extending over approximately the first 12 hours. The longer 11-hour periods of the early experiments do not reveal this feature, although it is highly probable that it actually occurred. The rise has been detected in all experiments in which observation of sufficient detail is available.

2. A rapid fall in rate follows the initial rise, extending from about the 12th–40th hour after isolation.

3. An interruption occurs in the falling phase, which may vary considerably in its general form. This stage of starvation is accompanied by the rapid yellowing of the leaves. It extends from about the 40th to 140th hour after isolation from the plant.

4. Concurrent with rapid browning of the leaves, a marked fall in the rate of carbon dioxide production occurs. Exudation of water, disorganization of the protoplast and the filling of the intercellular spaces with water all indicate that this phase may be considered as the " death " of the leaf-cells.

In all, over 70 samples of normal mature barley leaves have been examined, and the general features tabulated above have always been observed. The main variations occur in the form of carbon dioxide production during the yellowing phase. The aim of the analytical treatment, to be described here, can be conveniently re-defined as an attempt to discover to what extent the changes in rate of carbon dioxide production can be correlated with the exhaustion of carbohydrate metabolites.

(c) Carbohydrate Analyses

The method of removal of samples for analysis was different; experiments I–III were planned to give a general survey of carbohydrate exhaustion throughout starvation, and samples were removed for analysis up to the 240th hour after detachment of the leaves. In the other experiments, attention was focussed more especially on the initial stages; samples were analysed at 6-hourly intervals at first, and the final sample was removed after approximately 120 hours in experiment V, fig. 2. As can be seen from the figure, this was before the final rapid fall in rate of carbon dioxide production occurred, and the yellowing of the leaves was not quite complete.

By analytical methods the following sources of hexose carbohydrate have been detected in barley leaves:—glucose, fructose, sucrose, fructosan, starch.

Maltose was never found in leaf extracts, and this was so regardless of the presence or absence of starch.

The determinations carried out on leaves grown in 1932 and 1933 showed some well-marked differences, no doubt to be attributed in part to the widely contrasted seasons. Starch was never detected in the leaves of the former year, either by iodine staining, or estimation after hydrolysis by taka diastase. The iodimetric and Hagedorn-Jensen methods gave consistent agreement in the determination of sucrose, the increase in glucose corresponding closely with half the increase in total reducing sugars.

In the following season, 1933, starch was present in the leaves in considerable quantities, representing nearly 20% of the total hexose available in some leaves. A further difference was the variation in sucrose values, as determined by the iodimetric and Hagedorn-Jensen methods. This was so whether invertase or acid hydrolysis was used, a higher value being found for the increase in total sugar than that obtained by doubling the increase in glucose.

TABLE I—SUCROSE DETERMINATION IN BARLEY LEAVES OF THE SEASON 1932

| | Increase on hydrolysis mg sugar | | | |
| | 2·5% sulphuric acid | | Invertase | |
Extract No	Hagedorn-Jensen glucose + fructose	Iodimetric glucose	Hagedorn-Jensen glucose + fructose	Iodimetric glucose
1	142	69	139	70
2	155	72	153	79
3	63	33	61	31
4	107	56	—	—
5	36	19	34	18
6	120	59	—	—
7	110	53	114	57

The explanation of these observations is almost certainly the presence of small quantities of fructosan, which, being hydrolysed by acid and invertase, give rise to pure fructose under the conditions used in sucrose determination. Therefore, in presentation of results of these analyses, the difference between the iodimetric and Hagedorn-Jensen methods has been reported as fructosan.

The contrast between this observation and that of de Cugnac (1931) is most probably due to the exceptional nature of the 1933 growing season. Analysis of leaves during this season repeatedly gave total

carbohydrate contents nearly twice as great as those of the previous year.

TABLE II—SUCROSE DETERMINATION IN BARLEY LEAVES OF THE SEASON 1933

	Increase in hydrolysis mg reducing sugar			
	2·5% acid		Invertase	
Extract No	Hagedorn-Jensen glucose + fructose	Iodimetric glucose	Hagedorn-Jensen glucose + fructose	Iodimetric glucose
1	244	112	253	115
2	167	70	168	69
3	101	40	102	40
4	375	167	370	165
5	92	36	90	34
6	91	37	96	35
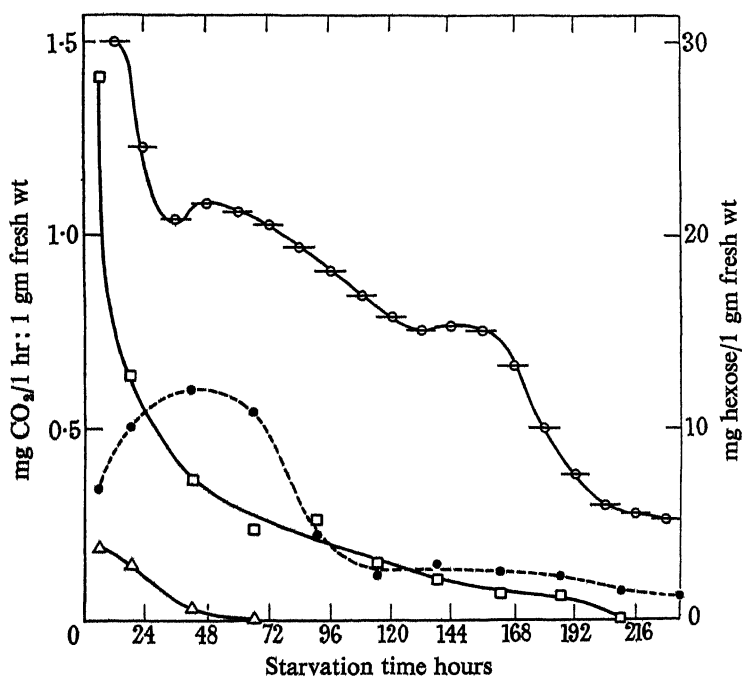
FIG. 3—Carbohydrate exhaustion and carbon dioxide production. Sucrose □ ; glucose ● ; fructose △ ; mean CO_2 ⊖

The analytical results of experiments I and V are summarized graphically in figs. 3 and 4. The carbohydrates are calculated to unit gm fresh

weight, and for sucrose, fructosan, and starch are given as mg hexose resulting from hydrolysis. The mean rates of carbon dioxide production, as calculated from the separate samples, are given in the figures for comparison.

Sucrose is the chief source of hexose in the leaves initially, forming 60–70% of the total carbohydrate present. All the experiments show
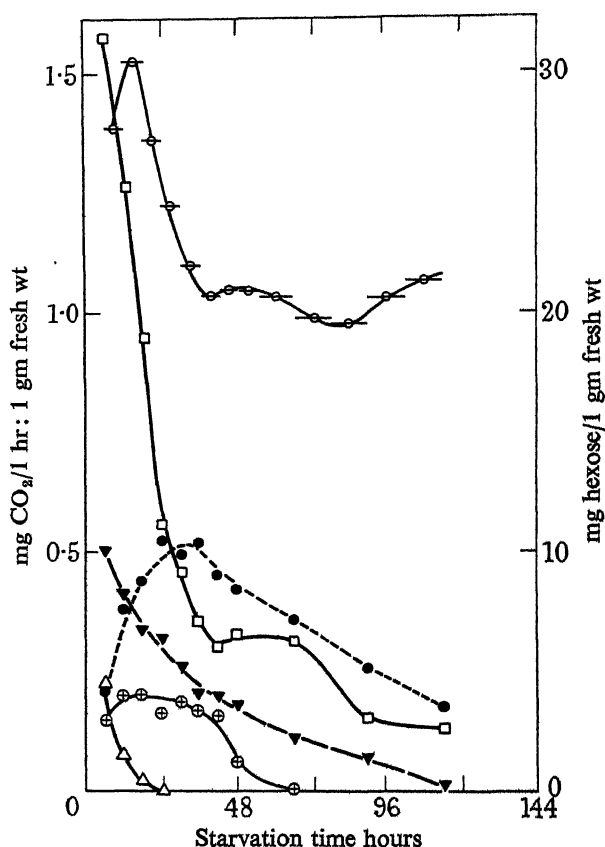
FIG. 4—Carbohydrate exhaustion and carbon dioxide production. △ fructose; ⊕ fructosan; ⊖ mean CO_2; ● glucose; ⊡ sucrose; ▼ starch

that on starvation a rapid fall in the concentration of this sugar occurs. Concurrently there is a complete disappearance of fructose, while the concentration of glucose rises at first, and later falls away to a low value. These general interrelationships of sucrose, glucose and fructose have been observed in every experiment of this type. The results of experiment V are further complicated by the presence of starch and fructosan as additional sources of hexose. Starch disappears slowly and continuously,

so that at the end of the experiment, when the leaves were 75% yellow, it could no longer be detected. The amount of fructosan present is approximately constant up to the 40th hour after isolation, and then a rapid exhaustion occurs, a concurrent interruption in the falling sucrose curve suggesting that a fructosan → sucrose transformation may take place.

3—CARBOHYDRATE CONCENTRATION AND RATE OF CARBON DIOXIDE PRODUCTION

A full examination of the preceding experimental records, and others of the same type, has shown that no simple quantitative relationship exists between the concentration of any individual carbohydrate and the rate of carbon dioxide production. None of the major variations in rate of CO_2 production can be correlated directly with concurrent changes of the individual sugars. Similarly, the concentration of free hexose (glucose + fructose) remains almost constant over the initial stages, when a rapid fall in rate of CO_2 production occurs.

The nature of the carbohydrate transformations suggests that each of the individual sources may make some special contribution to the sugar substrate for respiration. Accordingly the relationship between the rate of CO_2 production and exhaustion of total carbohydrate has been examined, figs. 5 and 6.

If the total carbohydrate concentration were controlling the rate of CO_2 production in a direct manner, it follows that the ratio of rate of production to concentration R/S should remain constant. These R/S ratios have been calculated from the data summarized above, and from other similar experiments. The mean rate of CO_2 production was divided by the mean concentration of carbohydrate, determined from analysis at the beginning and end of the appropriate period. In fig. 7 these values have been plotted against time.

An approach to constancy in the value is only realized over the first phases of carbohydrate exhaustion, later a steady increase is observed. The more detailed results of experiment V are of chief interest in this respect. They show that the rate of carbon dioxide production per unit total carbohydrate is constant from about the 12th to the 24th hour after isolation. This corresponds to the first stages of the marked fall in the rate of carbon dioxide production, which extends from the 12th to 40th hour after detachment. The evidence suggests, therefore, that this phase is causally connected with the exhaustion of the available sources of hexose estimated by analysis.

4—Production of Carbon Dioxide from Undetermined Sources

From the difference between successive carbohydrate analyses, it is possible to determine the rate of total sugar loss. Assuming complete oxidation of the sugar to carbon dioxide and water, the actual amount of carbon dioxide accounted for can then be calculated. This has been done for different experiments, and the results are summarized in figs. 8, 9, and 10.
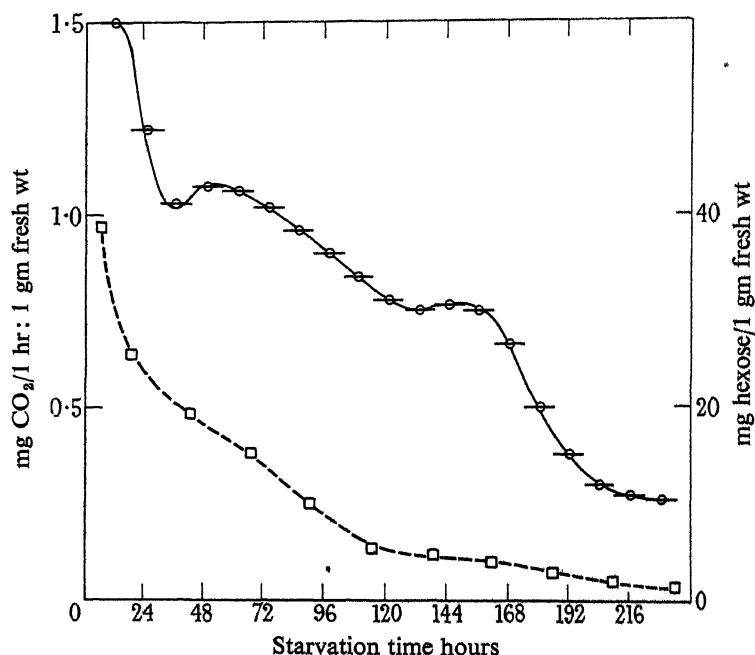
Fig. 5—Exhaustion of total carbohydrate (given as mg hexose) with starvation. Mean CO_2 ⊖; total carbohydrate □

From these figures it is clear that only in the first stages can the carbon dioxide be considered to arise solely from the normal sources of hexose estimated by analysis. Progressive with starvation time there is an increasing component arising from undefined sources (indicated by the dotted line in the figures). Consequently, throughout the yellowing phase, 40th → 140th hours, less than 25% of the total CO_2 produced can be accounted for by carbohydrate loss. In experiments II, III, and IV, carried out in the 1932 season, it was found that even over the first 12 hours the estimated hexose loss did not account for all of the carbon dioxide produced, experiment II, fig. 10.

The experimental data have shown that the production of carbon dioxide from the undetermined sources depends, primarily, upon the concentration of the available carbohydrate. In the different experiments a variety of initial concentrations were observed, and it was found that, with increasing initial concentration, the oxidation of the undetermined substrate was progressively retarded during starvation. The results are summarized in fig. 11, in which the mg of CO_2 arising from
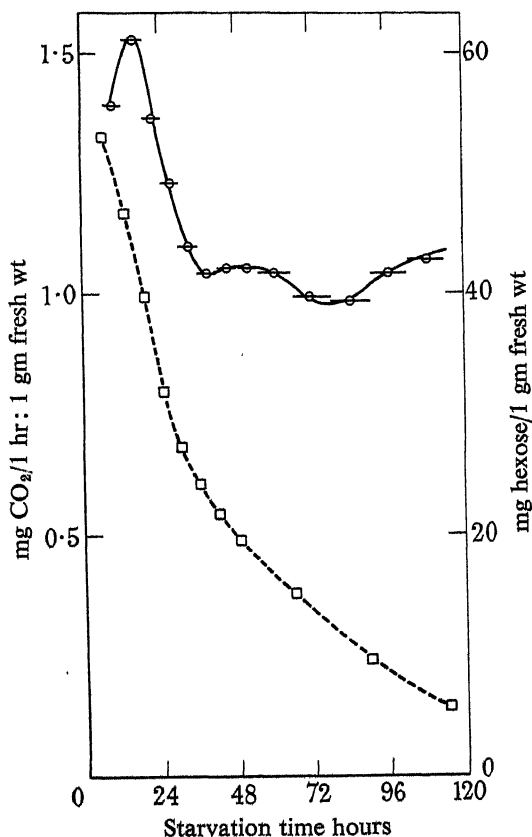
FIG. 6—Exhaustion of total carbohydrate with starvation. ⊖ mean CO_2; ☐ total carbohydrate

undetermined sources during first 12 hours are plotted against initial sugar concentration, for experiments I–V.

It seems clear that at higher sugar concentrations the oxidation of the other substrate is prevented, and a preferential oxidation of carbohydrate occurs with a production of carbon dioxide from this source only. Not only is this shown by a comparison of the different experiments, but also by the individual experiments themselves. Starvation of the leaves
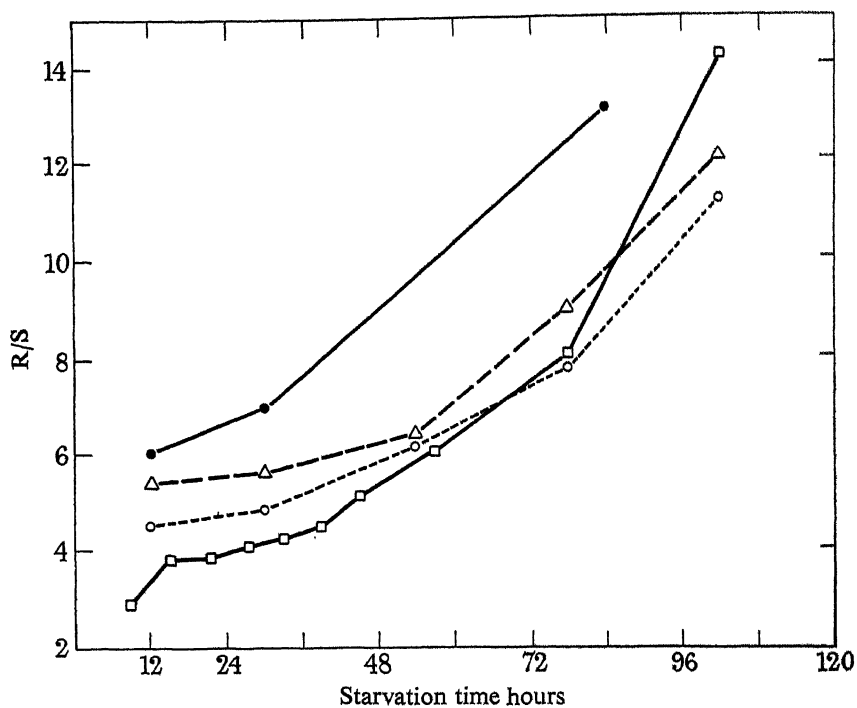
FIG. 7—Rate of carbon dioxide production per unit carbohydrate concentration during starvation. Experiment I, ○; II ●; III △; V □
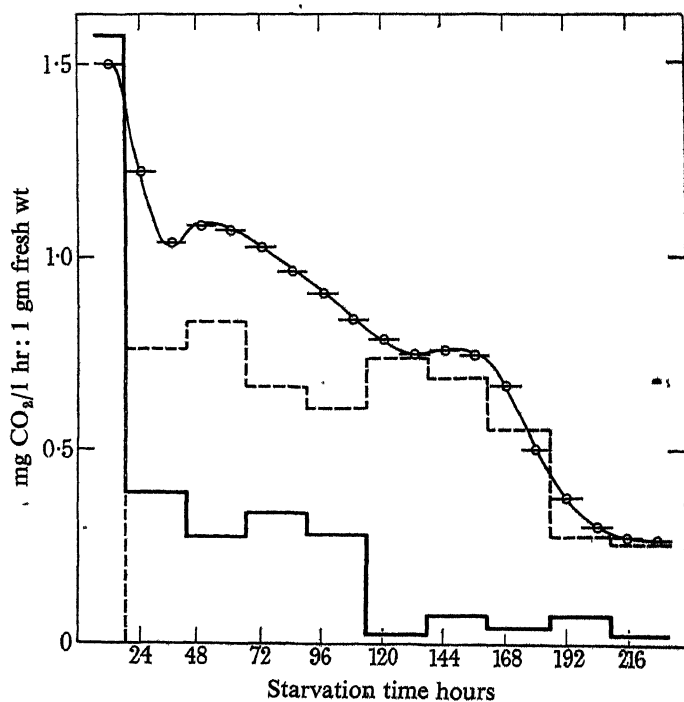
FIG. 8—Carbon dioxide equivalent to the loss of total carbohydrate experiment I. Mean CO_2 output ⊖; $CO_2 \equiv^t$ carbohydrate loss —; – – – CO_2 not accounted for by carbohydrate loss

leads directly to a reduction of the carbohydrate concentration, and this results in the production of carbon dioxide from undetermined sources.
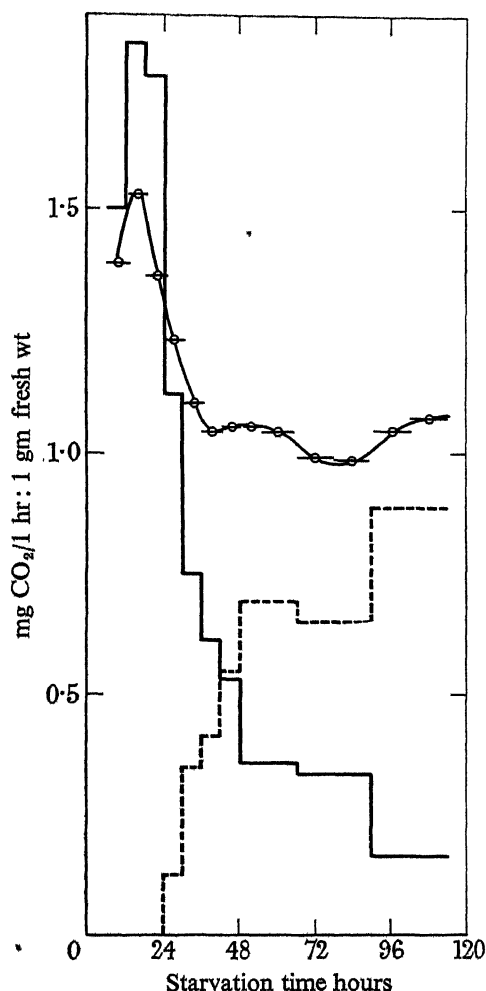
FIG. 9—Carbon dioxide equivalent to the loss of total carbohydrate, experiment V. $\ominus$ mean CO_2 output; — CO_2 $\equiv^t$ carbohydrate loss represents rate of carbohydrate loss calculated as mg CO_2, per hour, per 1 gm fresh weight; – – – CO_2 not accounted for by carbohydrate loss

5—Respiratory Quotient during Starvation

The demonstration of the active production of carbon dioxide from some source other than the decomposition of normal carbohydrate metabolites led directly to the measurement of the respiratory quotient
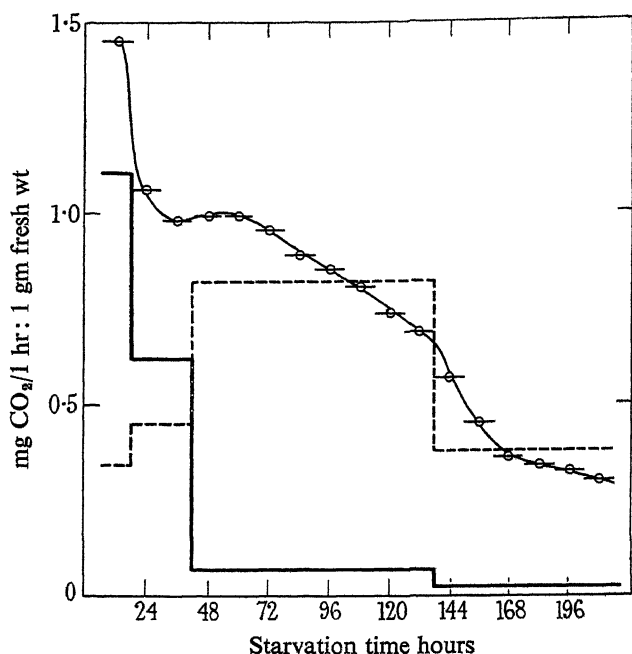
FIG. 10—Carbon dioxide equivalent to loss of total carbohydrate, experiment II. Mean CO_2 output $\ominus$; $CO_2 \equiv^t$ carbohydrate loss —; – – – CO_2 not accounted for by carbohydrate loss
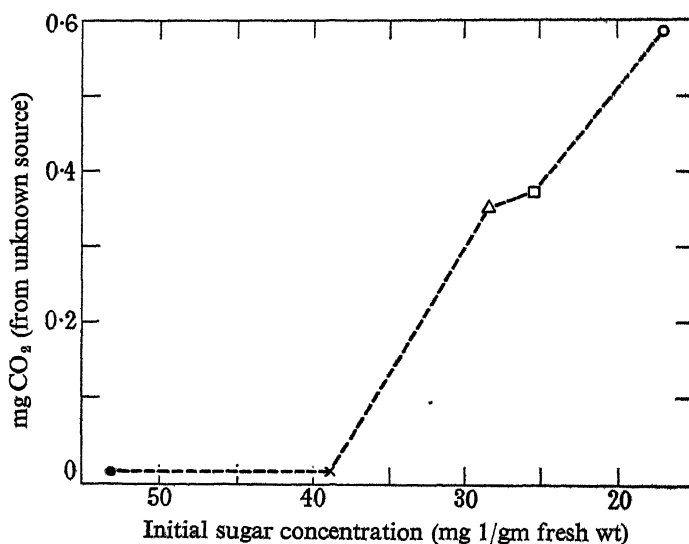
FIG. 11—Production of CO_2 from undetermined sources during first 12 hours' starvation. Experiment I ×; II □; III △; IV ○; V ●

during starvation. Three such experiments have given essentially similar results. One of them is of particular interest, experiment VII, in conjunction with the results of experiment V, already described. A random sample of 45 leaves was taken at the same time as those used for carbohydrate analyses, and this was used for the measurement of the quotient. By the use of the Haldane gas analysis apparatus, rates of carbon dioxide production and oxygen uptake were determined, under conditions of
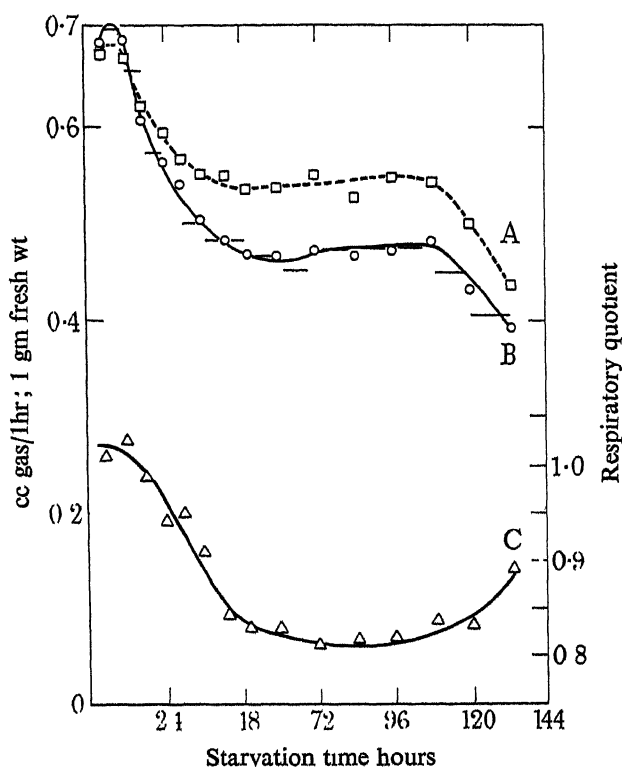
FIG. 12—Carbon dioxide production and oxygen uptake during starvation. Pettenkoffer, CO_2 —; Haldane, CO_2 ○; O_2 □; A, O_2 uptake; B, CO_2 output; C, R.Q.

starvation similar to those of the other experiment, so that a direct comparison of results is possible.

The observations are summarized graphically together with the calculated respiratory quotient, R.Q., in fig. 12.

Up to the 20th hour after detachment of the leaves a quotient very close to unity is recorded, and from the 20th to the 40th hour a rapid transition occurs to a value of about 0·82. This low value is maintained during the yellowing phase, and a rise is observed after 120 hours, con-

current with the final rapid fall in rate of CO_2 production. It is clear that this final rise in R.Q. is to be associated with rapid browning of the leaf. The condition of the majority of the cells at this phase indicates that the connection between these oxidations and normal cell respiration must be remote.

Chief interest is attached to the earlier stages, and a comparison of the above results with those of the carbohydrate analyses, experiment V, fig. 9, is of importance. The initial period with the R.Q. of unity corresponds closely with that in which all the carbon dioxide is accounted for by loss of carbohydrate. This is to be expected if carbohydrate is the only substrate undergoing oxidative breakdown. Similarly the transition to a low rate of sugar loss is accompanied by a falling R.Q., and the steady value corresponds to the phase at which most of the carbon dioxide arises from undetermined sources.

The observation that the production of carbon dioxide from unknown sources has a definite effect upon the respiratory quotient affords a clue as to the biochemical nature of this substrate. From a knowledge of their average composition, it is deduced that the respiratory quotient, during the normal oxidation of protein alone, should be approximately $0 \cdot 8$. Assuming protein to be the unknown source of carbon dioxide, it is possible to calculate from the analytical data of experiment V the quotient to be expected over the various periods during starvation. This has been done and the values are shown by a dotted line in fig. 13, together with the quotients actually observed with the sample taken at the same time.

A close similarity is apparent between the respiratory quotients observed and those calculated on the assumption that protein breakdown is involved. Such differences as exist may be due to the oxidation of different types of protein; little information is available concerning the constitution of the proteins present in foliage leaves. Furthermore, it is possible that other non-carbohydrate substrates may be breaking down concurrently. However, the evidence suggests that protein breakdown is one of the main reactions underlying carbon dioxide production during the later stages of starvation.

6—DISCUSSION

The foregoing experimental record describes the measurement of a number of processes primarily connected with leaf respiration. The results indicate that the respiratory mechanism is profoundly modified under conditions of prolonged starvation. While the normal carbo-

hydrate metabolites are maintained at a high level, oxidation of these only occurs. An exhaustion of the available carbohydrate causes a rapid fall in the rate of carbon dioxide production, and the results show that at subsequent stages the oxidative breakdown of protein may play an important part in leaf respiration.

The analytical data have shown that the falling rate of carbon dioxide output is more nearly related to the fall in total carbohydrate concentration, rather than to the exhaustion of any single sugar. It has been
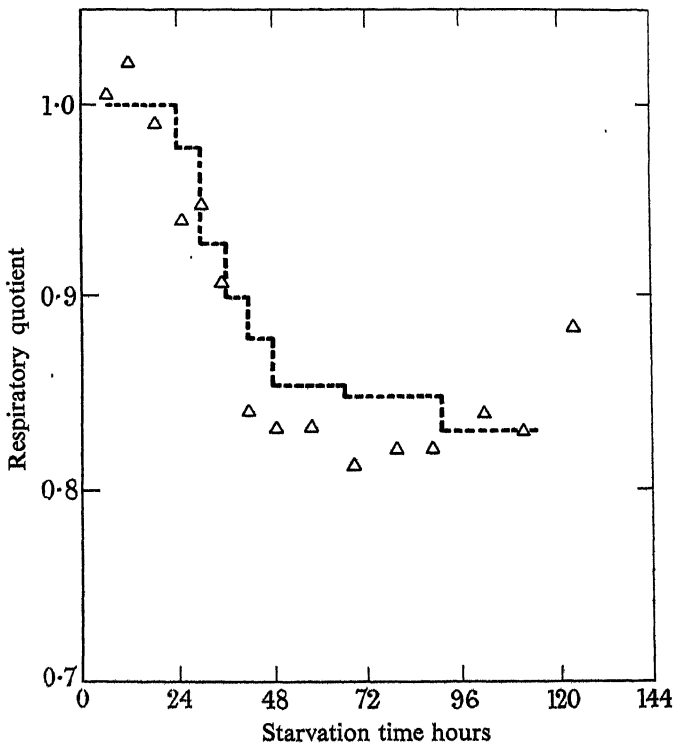
Fig. 13—Respiratory quotient, calculated from analytical data, experiment V, and observed, experiment VII. - - - Calculated; △ observed

suggested that each carbohydrate source may make a separate contribution to the respiratory substrate. Blackman (1928) has indicated that one of the stages in carbohydrate breakdown may involve a change of the normal stable hexose to more labile " heterohexose." In the light of Haworth's work (1929) on the structure of the sugars, this change has been interpreted by Onslow, Kidd, and West (1931) as a change from the pyranose to furanose structure. It seems likely, therefore, that the different carbohydrate sources may influence the rate of carbon dioxide

production by the formation of the furanose substrate by direct hydrolysis. When sucrose, glucose, and fructose form the only sources of carbohydrate experiments I–IV, it is probable that two main sources of labile furanose are available, one by direct hydrolysis of sucrose and the other by the activation of normal pyranose. The breakdown of sucrose may be regarded as involving the following linked reactions:—

$$\text{Sucrose} \;(K_1) \nearrow \overset{(K_2)}{\text{fructo-furanose}} \to \text{glycolysis products.}$$

$$\searrow \overset{(K_3)}{\text{gluco-pyranose}} \to \text{labile product} \to \text{glycolysis products.}$$
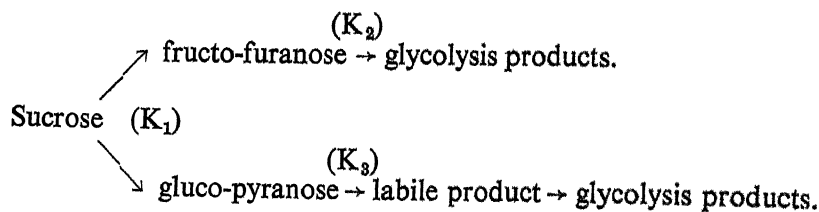
Onslow has suggested from her own (1931) and collected data (Onslow, Kidd, and West (1931)), that in such a system fructo-furanose may be preferentially respired, leading to an accumulation of normal pyranose in respiring plant tissues. In all the experiments of this investigation, it has been found that although the furanose component disappears much more rapidly, the pyranose also disappears, but at a slower rate. The exhaustion of pyranose $\left(\text{glucose} + \text{fructose} + \dfrac{\text{sucrose}}{2}\right)$ and furanose $\left(\dfrac{\text{sucrose}}{2}\right)$ calculated from the data of experiment I is shown in fig. 14.

It is apparent that the rate of loss of pyranose is much slower than that of furanose; the former disappears slowly and steadily up to 120 hours after isolation, while furanose sugar is practically exhausted after 48 hours. This has been shown consistently by experiments of this type. The experimental evidence suggests that the action velocities K_1, K_2, K_3 of the previous diagram must have an order of magnitude as follows:—

$$K_2 > K_1 > K_3.$$

It seems likely that these are the dynamic conditions underlying the characteristic sugar transformation, experiments I–IV, rapid sucrose hydrolysis occurring concurrently with the disappearance of all fructose and the accumulation of glucose. After the sucrose supply has fallen below a certain level, K_1 is no longer greater than K_3, and the slow fall of glucose concentration is observed.

The data of experiment V are more complicated; the presence of starch and fructosan may influence the supply of furanose, either directly or by transformation to sucrose. There is some evidence that this transformation to sucrose does occur, and, assuming that starch and

fructosan are equivalent to sucrose, it has been shown that pyranose loss is slower than furanose.

The above outline of the mechanism of sugar transformation depends upon the assumption that the total carbohydrate loss is to be attributed to respiratory breakdown. In experiment V there is evidence that this is not so; during the early stages, the loss of sugar more than accounts for the carbon dioxide produced, assuming complete oxidation. The fate of the excess sugar remains doubtful—partial oxidation, oxidative anabolism to non-sugar products, or condensation may account for it.
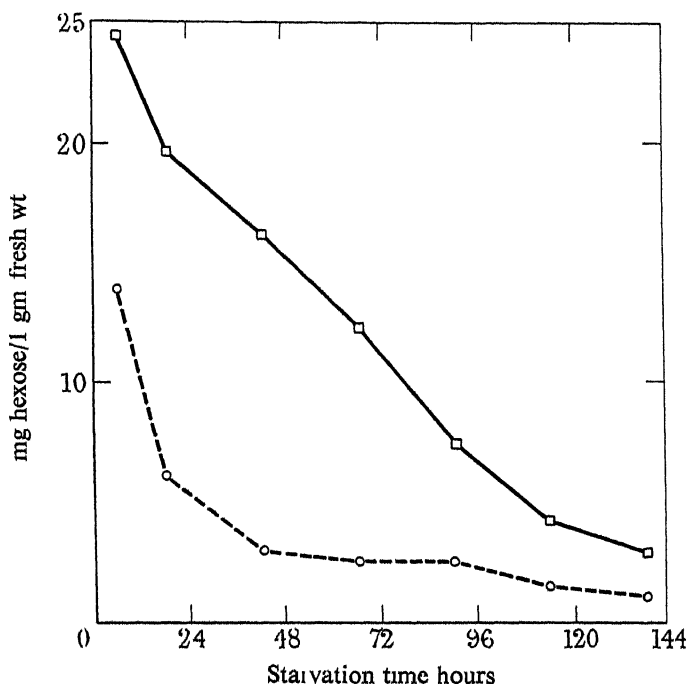
FIG. 14 —Exhaustion of pyranose and furanose sugar in starving leaves. □ pyranose; ○ furanose

Experiments of the same type as those described with barley leaves have been carried out with isolated broad bean leaves (Sutton's Exhibition Long Pod). Carbohydrate analyses showed that, up to about 30 hours after isolation, the total carbon dioxide output was accounted for by carbohydrate loss, and over a similar period an R.Q. close to unity was observed. During this time the rate of carbon dioxide production was maintained at a high level, and subsequently a marked fall occurred, coincident with a change in R.Q. to a lower value of approximately 0·93. At all subsequent periods the analyses indicated that the loss of

normal carbohydrate metabolites was not equivalent to the total carbon dioxide produced. There is every indication that the metabolism of isolated barley and bean leaves under starvation conditions is essentially similar. The main difference is the rate at which the changes occur in the different types of leaf; carbohydrate exhaustion, and subsequent oxidation of a non-sugar substrate, occurring more slowly in bean leaves.

In relation to these observations, the results of Deleano (1912) are of interest. Using isolated vine leaves under starvation conditions at 18–23° C, he found that the loss of carbohydrate was equivalent to the total carbon dioxide produced for about the first 100 hours. It is clear that the rate at which changes associated with starvation occur depends markedly upon the leaf type. Blackman (1908) and Godwin and Bishop (1927) have described the carbon dioxide production of cherry laurel leaves during starvation. Depending on their metabolic age, browning and death of these leaves does not occur until upwards of 20 days after isolation, compared with the 5–6 days of barley, and 7–8 days of bean leaves. The actual form of the carbon dioxide emission curves for cherry laurel leaves, given by the above authors, suggests that some of the important changes may be similar to those described here. Further complication may occur, owing to the production of free HCN during yellowing (Godwin and Bishop, 1927), and the effect of this on hydrolytic enzymes (Hanes and Barker, 1931).

Deleano (1912) observed that a rapid loss of protein nitrogen occurred in vine leaves after the exhaustion of the carbohydrates. The nitrogenous metabolism of starving leaves has been investigated more recently by Chibnall (1924) and Mothes (1926). The general results of both this work and that on starved seedlings show that under conditions of carbohydrate shortage amino acids, formed from protein hydrolysis, undergo decomposition with the production of amides or ammonia. Evidence from analytical results and measurement of the respiratory quotient have been presented which suggest that protein katabolism is one of the important reactions concerned with carbon dioxide production in leaves after prolonged starvation. Further discussion of this problem is postponed until other experimental results have been described.

SUMMARY

A study of carbon dioxide production, oxygen uptake, and carbo-hydrate concentration in starving barley leaves had led to results which may be summarized as follows:—

Only during the initial stages can carbohydrate be considered as the only substrate undergoing oxidative breakdown.

A rapid fall in rate of carbon dioxide output is associated with carbohydrate exhaustion, and, subsequent to this, oxidation of other non-carbohydrate substrates occurs.

It is highly probable that protein katabolism is one of the important reactions concerned with carbon dioxide production during the later stages of starvation.

The nature of the sugar transformations is discussed briefly together with the relation of these observations to work on other types of leaf.

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END OF THE ONE HUNDRED AND FOURTEENTH VOLUME (SERIES B).